IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

APPLICANT: Mountz, *et al.*	**ART UNIT: 1644**
FILED: May 15, 1998	
SERIAL NO.: 09/079,83	**EXAMINER: Tung, M.**
FOR: Fas Ligand Expressing Antigen Presenting Cells for Tolerance Induction	**DOCKET: D6005**

Assistant Commissioner of Patents
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ATTENTION: Board of Patent Appeals and Interferences

APPELLANT'S BRIEF

This Brief is in furtherance of the Notice of Appeal filed in this case on January 31, 2000. The fees required under 37 C.F.R. §1.17(f) and any other required fees are dealt with in the accompanying TRANSMITTAL OF APPEAL BRIEF.

In accordance with 37 C.F.R. §1.192(a), this Brief is submitted in triplicate.

INDEX OF SUBJECT MATTER

I. REAL PARTY IN INTEREST

The real party in interest is the University of Alabama at Birmingham Research Foundation.

II. STATUS OF THE CLAIMS

Originally claims 1-17 were filed with this Application. Claims 10-15 were withdrawn from consideration. Claims 2, 7, 17 were canceled, and claims 1 and 16 have been amended. Claims 8 and 9 were objected to. The pending claims 1, 3-6, and 16 are being appealed, of which claims 1 and 16 are independent claims.

III. STATUS OF AMENDMENTS

Claim 1 was amended in the Response to the Office Action of October 27, 1998. Claim 16 was amended in the Response to the Office Actions of April 21, 1999. Subsequent to the final rejection mailed October 29, 1999, Applicants submitted a Response After

Final which canceled claims 17, and added claims 18 and 19. In an Advisory Action mailed December 8, 1999, claims 18 and 19 were not entered into the record. All claims as pending are shown in Appendix A.

IV. STATEMENT OF RELATED APPEALS AND INTERFERENCES

To Applicant's knowledge, there are no pending related appeals or interferences which will directly affect or be directly affected by the present appeal.

V. SUMMARY OF THE INVENTION

The present invention is drawn to a method of inducing systemic tolerance to a viral or alloantigen using antigen presenting cells that express Fas ligand and the antigen, wherein said antigen presenting cells induce apoptosis of Fas-positive T cells directed towards said antigen resulting in induction of systemic tolerance to said antigen (Specification, page 9, line 3-page 10, line 5). The

present invention is also drawn to a method of using Fas ligand-expressing antigen presenting cells from a graft to create immune-privileged sites so as to decrease rejection of a graft (Specification, page 24, line 19-page 25, line 10).

VI. ISSUES

35 U.S.C. §103

Whether claims 1 and 3-6 are unpatentably obvious under 35 U.S.C. §103(a) over **Bellgrau** et al. in view of **Süss** et al.

Whether claims 1 and 3-6 are unpatentably obvious under 35 U.S.C. §103(a) over **Bellgrau** et al. in view of **Schuler** et al.

Whether claim 16 is unpatentably obvious under 35 U.S.C. §103(a) over **Bellgrau** et al. in view of **Süss** et al.

Whether claim 16 is unpatentably obvious under 35 U.S.C. §103(a) over **Bellgrau** et al. in view of **Schuler** et al.

VII. GROUPING OF CLAIMS

The rejected claims do not stand or fall together. Applicant considers claims 1, 3-6, and 16 lie in two embodiments of the present invention. Claims 1 and 3-6 are drawn to a method of inducing systemic tolerance to a viral antigen, an autoantigen or an alloantigen using Fas ligand-expressing antigen presenting cells. Claim 16 is drawn to a method of creating immune-privileged sites so as to decrease transplant rejection.

VIII. ARGUMENTS

The Rejection Under 35 U.S.C. §103

In the Final Office Action mailed October 29, 1999, the Examiner maintained the rejection of claims 1 and 3-6 under 35 U.S.C. §103(a) as being unpatentable over **Bellgrau** et al. in view of **Süss** et al. This rejection is respectfully traversed.

Bellgrau et al. teach a method of inhibiting T-lymphocyte-mediated immune responses by providing a recipient animal with Fas ligand or cells expressing Fas ligand. **Bellgrau** et al. do not teach the use of antigen presenting cells to express Fas ligand in said method.

Süss et al. teach that a sub-population of dendritic cells express Fas ligand and induce apoptosis of $CD4^+$ T cells which results in the down regulation of the immune response. The Examiner argued that one of ordinary skill in the art at the time the invention was made would have been motivated by the combined teaching of these two references and have reasonable expectation of success to use Fas ligand-expressing antigen presenting cells in methods of antigen-specific immunosuppression as disclosed in the instant invention.

However, in determining whether such a suggestion can be fairly gleaned from the prior art, the full field of the invention must be considered as a person of ordinary skill is charged with knowledge of the entire body of technological literature, including that which might lead away from the claimed invention. References

that teach away from the instant invention, i.e. showing Fas ligand expression did not lead to immunosuppression and prevention of graft rejection but rather induce an inflammatory response and accelerated graft rejection, were provided in the Specification (page 6, lines 16-20) and discussed in the Response After Final (**Kang** et al. and **Chen** et al.; enclosed). More specifically, high expression of Fas ligand can lead to immune cell infiltration and inflammation instead of immunosuppression. For example, **Kang** et al. disclosed that Fas ligand expression on pancreatic islets results in neutrophilic infiltration and accelerated graft rejection. **Chen** et al. disclosed subcutaneous injection of stably transfected colon carcinoma cells that express Fas ligand results in neutrophils activation and rejection of the cancer cells. Thus, expression of Fas ligand does not always inhibit immune responses. The fact that Fas ligand expression can lead to enhancement as well as inhibition of immune responses indicates that the regulatory function of Fas ligand is more complex and varies between different experimental and in vivo settings.

The effect of Fas ligand expression on an immune response also depends on the presence or absence of other regulatory factors in that particular site (**Chen** et al., page 1715, left

column, lines 17-20). **Bellgrau** et al., however, only show results with soluble Fas ligand and Fas ligand expressed on islet cells, which are not antigen presenting cells. **Süss** et al. only show data from in vitro culture. It is well known to a person hainvg ordinary skill in this art that one cannot always equate in vitro data to in vivo results. The differences between in vitro and in vivo situations becomes more important in view of the fact that **Chen** et al. teach that the in vivo microenvironment and the presence of secondary factor play an important role in regulating the effect of Fas ligand expression (see abstract; page 1715, left column, lines 17-20; page 1715, middle column, lines 1-4). Therefore, the combined teaching of **Bellgrau** et al. and **Süss** et al. does not address the potential problem of inflammation induced by Fas ligand expression, and the data in the combined references do not necessarily lead to the conclusion that Fas ligand expression on antigen presenting cells would lead to immunosuppression in transplantation or autoimmunity in vivo.

The Examiner did not respond to the argument Applicants made with regard to the cited references in the Response After Final. Applicants respectfully submit that in view of the cited references that lead away from the claimed invention, and secondly

that there is no teaching or suggestion in the combined teaching of **Bellgrau** et al. and **Süss** et al. that indicate the use of Fas ligand as disclosed in the instant invention would not lead to stimulation of immune response, the present invention is not *prima facie* obvious to one skilled in the art at the time the invention was made.

Moreover, there are aspects of Applicants' claimed invention which are neither taught nor suggested in the combined teaching of **Bellgrau** et al. and **Süss** et al. More specifically, the use of Fas negative antigen presenting cells derived from the mutant *lpr/lpr* mice, and the use of a two virus system to achieve a very high level of Fas ligand expression (specification page 10, line 6-page 11, line 2) are not disclosed by the cited references.

In the Final Office Action mailed October 29, 1999, the Examiner maintained the rejection of claims 1 and 3-6 under 35 U.S.C. §103(a) as being unpatentable over **Bellgrau** et al. in view of **Schuler** et al. This rejection is respectfully traversed.

Bellgrau et al. teach a method of inhibiting T-lymphocyte-mediated immune responses by providing a recipient

animal with Fas ligand or cells expressing Fas ligand. **Bellgrau** et al. do not teach the use of antigen presenting cells to express Fas ligand in the method.

Schuler et al. is a review article that only cited the reference of **Süss** et al. (page 320, right column, paragraph 2) to suggest tolerance induction in transplantation and autoimmunity by Fas ligand-expressing dendritic cells. **Schuler** et al. did not demonstrate a method of inducing antigen-specific systemic tolerance by administering antigen presenting cells expressing Fas ligand and the antigen.

Hence, the combined teaching of **Bellgrau** et al. and **Schuler** et al. is essentially the combined teaching of **Bellgrau** et al. and **Süss** et al. As discussed above, Applicants respectfully submit that in view of the literature cited by the Applicants and discussed above which leads away from the claimed invention, and secondly since there is no teaching or suggestion in the combined teaching of **Bellgrau** et al. and **Schuler** et al. that indicate the use of Fas ligand as disclosed in the instant invention would lead to immunosuppression rather than stimulation of immune response, the

claims 1 and 3-6 is not *prima facie* obvious under 35 U.S.C. §103(a) to one skilled in the art at the time the invention was made.

In the Final Office Action mailed October 29, 1999, the Examiner maintained the rejection of claims 16 under 35 U.S.C. §103(a) as being unpatentable over **Bellgrau** et al. in view of **Süss** et al., and unpatentable over **Bellgrau** et al. in view of **Schuler** et al. These rejections are respectfully traversed.

Claim 16 is drawn to a method of creating immune-privileged sites in an individual so as to decrease rejection of a graft by introducing Fas ligand into antigen presenting cells derived from the graft. The teaching of **Bellgrau** et al., **Süss** et al. and **Schuler** et al. were discussed *supra*.

Applicants respectfully submit that in view of the literature cited by the Applicants and discussed above which teaches away from the claimed invention, the references in **Süss** et al. that contribute Fas ligand expression in Sertoli cells and cells of the anterior chamber of the eye to the creation of immune-privileged sites at those tissues, and the mere speculation in **Schuler** et al. that

Fas ligand-expressing dendritic cells may induce tolerance in transplantation do not enable one skilled in the art to successfully produce the claimed invention. As discussed *supra*, Fas ligand expression does not necessarily lead to immunosuppression and prevention of graft rejection but rather can induce an inflammatory response and accelerated graft rejection. Clearly, the effects of Fas ligand expression can vary greatly between different experimental settings *in vivo*. Thus, teaching with concrete data in a similar experimental situation is critical for one skilled in the art to produce the claimed invention. However, there are no data in the combined cited references that show the effect of Fas ligand-expressing antigen presenting cells on graft survival *in vivo*.

In contrast, the instant invention disclosed detailed examination of alloantigen specific T cell tolerance using antigen specific transgenic mice (Examples 16-17). Hence, Applicants respectfully submit that the combination of the cited references would not provide a person having ordinary skill in this art with the requisite motivation nor expectation of successfully producing Applicants' claimed methods. The invention as a whole is not *prima*

facie obvious to one of ordinary skill in the art at the time the invention was made, as evidenced by contrary references.

For the foregoing reasons, Applicant respectfully requests that the decision of the Examiner should be reversed, and that claims 1, 3-6, and 16 be allowed.

Respectfully submitted,

Date: Feb 9, 2000

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CLAIMS ON APPEAL

1. A method of inducing systemic tolerance to an antigen in an individual in need of such treatment, comprising the step of:

administering antigen presenting cells to said individual, wherein said cells express Fas ligand and said antigen, wherein said antigen presenting cells induce apoptosis of Fas-positive T-cells directed towards said antigen resulting in said induction of systemic tolerance to said antigen.

3. The method of claim 1, wherein said antigen is selected from the group consisting of the adenovirus antigen, a viral antigen, an adeno-associated viral antigen, an autoantigen, and an alloantigen.

4. The method of claim 1, wherein said individual has an autoimmune disease.

5. The method of claim 4, wherein said autoimmune disease is selected from the group consisting of diabetes, multiple sclerosis, rheumatoid arthritis, thyroiditis, Grave's disease, systemic lupus erythematosus.

6. The method of claim 1, wherein said individual has had an organ transplant.

8. The method of claim 1, further comprising the step of delivering to said antigen presenting cells a gene to inhibit apoptosis.

9. The method of claim 8, wherein said gene to inhibit apoptosis is crmA.

16. A method of creating immune-privileged sites in an individual so as to decrease rejection of a graft, comprising the steps of:

extracting antigen presenting cells from donor organ tissue;

introducing Fas ligand into said antigen presenting cells to produce Fas ligand-expressing antigen presenting cells expressing an antigen specific to said graft;

introducing said Fas ligand-expressing antigen presenting cells expressing an antigen specific to said graft to said individual prior to and during said grafting procedure; wherein said Fas ligand-expressing antigen presenting cells expressing an antigen specific to said graft create said immune-privileged site at the site of said grafting procedure to prevent rejection of said graft in said individual.

Mini R view

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Dendritic Cells: From Ignored Cells to Major Players in T-Cell-Mediated Immunity

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Key W rds
Dendritic cells
Antigen-presenting cells
T-cell-mediated immunity
Vaccination
Tolerance

Abstract
Dendritic cells form a system of leukocytes specialized to stimulate resting T cells in vivo. Dendritic cells are crucial for the initiation of primary immune responses of both helper and cytotoxic T lymphocytes, and thus act as 'nature's adjuvant'. The manifold specializations underlying this in vivo immunostimulatory function are becoming increasingly clear. Methods have been developed to generate large numbers of dendritic cells from hematopoietic precursors in vitro. These techniques now allow molecular studies as well as the use of antigen-charged dendritic cells to vaccinate patients against tumors (e.g. B-cell lymphoma or melanoma) and infection (e.g. HIV-1). Recent data suggest that besides the classical immunostimulatory dendritic cells which belong to the myeloid lineage, there exist regulatory dendritic cells related to the lymphoid lineage. These lymphoid-derived dendritic cells which at least in part express Fas-ligand appear to be involved in the induction of central as well as peripheral tolerance, and in the future might allow a novel approach to induce tolerance in transplantation, autoimmunity, and allergy.

Dendritic cells (DC) are specialized for presenting antigens to T cells in vivo, and are currently in the center of immunological research [1]. Here, we attempt to briefly review the emergence of the DC concept and will also outline some recent developments.

The Dendritic Cell System

In 1973, Steinman and Cohn [2] described a novel, scarce cell type in murine spleen and called it 'dendritic cells' (DC), as the cells displayed several types of processes including highly motile cytoplasmic sheets (so-called 'veils'). DC were soon found in other lymphoid organs (lymph node, thymus) as well as afferent lymph. Based on in vitro and in vivo studies, Steinman postulated that DC represent a subset of MHC class II positive, yet lineage marker negative leukocytes distinct from macrophages, and specialized to stimulate naive T cells and thus to initiate primary immune responses. Despite accumulating elegant experimental data, the novel concept of the DC was not accepted for many years likely because of three major facts. First, only few groups were studying DC as the isolation of these scarce (1%) cells from lymphoid tissues was demanding, and DC-specific antibodies were at that time not available for monitoring or positive enrichment. Secondly, all but a few immunologists stuck to the traditional view that macrophages were not only professional phagocytes and scavengers but

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also the major professional antigen presenting cells. Thirdly, there was indeed a problem with the DC concept in that it was difficult to understand how rare DC located in secondary lymphoid tissues like spleen or lymph node could be critical for induction of T-cell-mediated immunity to small amounts of antigen deposited in the periphery (e.g. body surfaces like skin).

In 1985, studies on murine epidermal Langerhans' cells (LC) suggested a solution to this puzzle as they unravelled that DC existed in two maturational stages in nonlymphoid versus lymphoid tissues ('concept of DC maturation'). Initially it was shown that LC can mature in vitro into potent immunostimulatory DC virtually identical to those isolated from secondary lymphoid organs, implying that LC constitute direct yet immature precursors of lymphoid DC [3]. Then it was demonstrated that LC, i.e. immature DC, were specialized for antigen processing ('antigen processing mode') whereas following maturation DC had down-regulated their processing capacity but had developed potent T-cell sensitization capacity ('T-cell stimulatory mode') [4–6]. Finally, it became clear that the maturation was dependent on cytokines such as IL-1 and GM-CSF [7]. In vivo studies indicated that LC matured in vivo as well while emigrating from the epidermis into afferent lymphatics [8]. Further evidence for the concept of DC maturation came from studies on DC at other sites than skin, for example the lung [9], the interstitium of solid organs [10] as well as DC in blood [11]. From these and other studies the concept finally evolved that a *system* of dendritic cells is specialized for presenting antigens in vivo, and to act as nature's adjuvant for helper and killer T-cell formation [12].

In 1992 a third era of DC research began when methods were described to generate substantial numbers of murine and human DC in vitro from proliferating hematopoietic precursors under the aegis of GM-CSF [13,14]. A series of studies followed including reports on the generation of human DC from proliferating, rare CD34+ as well as more committed, nonproliferating, CD14+ precursors in peripheral blood of normal adults [15–18]. The availability of methods to generate DC in vitro has eliminated the major obstacle in DC research, i.e. that only few DC could be isolated despite tricky enrichment procedures. There are now also useful monoclonal antibodies available in mouse [12] and man [19] that are rather DC-specific. These new techniques have opened up the field of DC biology to many researchers, and will allow a molecular understanding of DC function as well as the use of DC for immunotherapy.

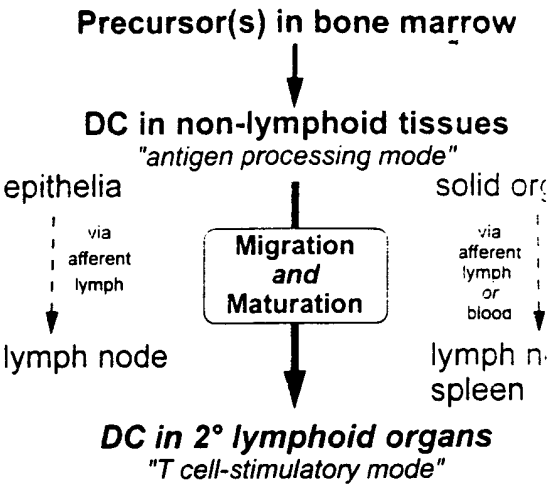

Fig. 1. The life cycle of dendritic cells. See text for det

Induction of Immunity by Dendritic Cells

DC capture antigen at peripheral sites and then to T areas in secondary lymphoid organs, where th antigen-specific T cells from the circulating pool a tize them [12]. DC display manifold specializat their task as 'nature's adjuvant' that are operative a cessive stages of antigen processing, migration a sensitization (fig. 1). It is this unique array of fu properties regulated in a temporally coordinated during a maturation process that has rendered DC to other antigen presenting cells in inducing (es primary immunity. The basic mechanisms by whic ture DC perform effective capture of exogenous and antigen processing for MHC class II loading rather well understood [20]. Under certain conditi appear capable of loading their MHC class I mole well with peptides derived from exogenous antigen dition to loading with peptides derived from (e.g. vi teins synthesized in the cytosol via the 'classica pathway') [20]. The respective mechanisms ('alt class I pathway') are, however, largely unknown in to MHC class II loading pathways. Immature DC up soluble antigens in the fluid phase as effectively gen-specific B cells (e.g. tetanus toxoid at 10^{-10} M constitutive *macropinocytosis* as unravelled by Sa al. [21]. Macropinocytosis is a special type of actin- ent fluid phase endocytosis, which enables a DC to half of their own volume within just 1 h. Another nism is *receptor-mediated endocytosis* via coat

which allows effective uptake of antigens at about 100-fold lower concentrations. The immature DC of stratified epithelia, i.e. LC, display an additional unique uptake pathway via the so-called Birbeck granules which may combine features of the macropinosome and coated pit pathway. One example for receptor-mediated antigen capture is the uptake of antigen-IgG complexes via FcγRII (CD32) (e.g. uptake of tetanus toxoid at 10^{-12} *M*) [16, 21] and of antigen-IgE complexes via the recently described FcεRI on DC [22]. As high-affinity antibodies are the result of an immune response, this pathway cannot be relevant for primary responses but might be important for the amplification of ongoing immune responses such as in allergy. In contrast to FcR the expression of multilectin 'pattern-recognition receptors' such as the recycling mannose receptor (8 contiguous, calcium-dependent or C-type lectin domains) [21] or the recently cloned DEC-205 (10 contiguous C-type lectin domains) [23] allows DC to bind and effectively take up mannosylated proteins (present on the surface of many microbes) even in the absence of antibodies and preexisting immunity. DEC-205 and related receptors might turn out useful tools to target antigens to DC for induction of immunity as well as tolerance (see below). Immature DC such as LC display also phagocytic activity and thus can take up particulate antigens such as bacteria as well [24]. Following uptake, antigens are finally delivered to HLA-DM+ late endosomal/lysosomal compartments (termed MIICs) where antigens are processed by proteases into peptides and loaded onto newly synthesized MHC class II molecules [20]. The resulting stable MHC-peptide complexes (i.e. TCR ligands) are then shuttled to the cell surface, and appear to be retained over several days for later presentation to T cells. As far as known, these in vitro observations reflect well what happens in vivo. Immature DC such as LC in vivo in the resting state have little MHC class II on their surface yet possess MIICs [25]. Antigen processing (i.e. uptake and proteolytic digestion of antigens, transient MHC II synthesis) is likely switched on by inflammatory cytokines such as IL-1 β (see below), and is short-lived as LC 24 h after such a stimulus have shut off antigen processing and display virtually all MHC class II molecules on the cell surface [6]. Lanzavecchia's group has uncovered several additional functional aspects by studying a model of immature DC that are not isolated from tissues but generated in vitro from CD14+ monocytes by using a combination of GM-CSF + IL-4. The studies suggest that exposure of DC precursors to certain cytokines such as GM-CSF + IL-4 may freeze the state in which DC can capture antigen in bulk, a state that is otherwise short-lived [16, 21]. Interestingly, even then the antigen capture mode can still be shut off by inflammatory stimuli (TNF-α, IL-1, LPS) as well as the CD 40 ligand. These stimuli cause a loss of macropinocytosis, a down-regulation of mannose and Fc receptors, a loss of intracellular MCIIs and a simultaneous increase in cell surface MHC II, whereby specific MHC/peptide complexes are preferentially formed at high density (about 4×10^6 per cell). As previously described for LC maturation, the inducing stimuli also cause a transient increase of MHC II synthesis that is shut off within 16 h. It was also found that immature DC, at least in this model, besides MIIC contained a second MHC class II+ compartment represented by early endosomes containing mature MHC class II molecules internalized from and recycled back to the cell surface. Interestingly, this recyling compartment like the MIIC was lost upon exposure to maturation-inducing stimuli. The biological relevance of the recycling compartment is unclear at present, notably as it appears that most of the MHC-peptide complexes are generated in the MIICs by binding to newly synthesized MHC class II molecules, and no studies on primary DC directly isolated from tissues are available. One possibility is, however, that peptides generated under less denaturing and proteolytic conditions in early endosomes and loaded on recycling MHC class II molecules might generate a different set of T-cell epitopes. One might even speculate that recycling MHC class II molecules continuously tolerize to self peptides when presented on certain subsets of DC (see below). This in vitro model of DC maturation has also shown that the inflammatory stimuli do not only regulate antigen processing but increase adhesion and costimulatory molecules needed for stimulation of naive T cells as well and induce CD44 variants implicated in migration [16]. This lends further support to the current view that inflammatory cytokines released upon antigen deposition ('danger') induce immature DC to leave their resting state, and to undergo maturation, i.e. a cascade of coordinately regulated irreversible events consisting of antigen capture and processing followed by up-regulation of adhesion and costimulatory molecules that is completed during migration and homing to T areas of lymphoid organs. Indeed, recent animal studies have shown that TNF-α, IL-1, and LPS promote the maturation and emigration (and even depletion) of DC from non-lymphoid tissues [24]. There are now three routes known by which DC can reach the lymphoid organs. DC in epithelia such as skin, airways, or gut acquire antigens deposited locally and migrate via afferent lymph to nodes. DC in the interstitium of solid organs like heart and kidney capture local antigens and then migrate either via lymph to draining nodes or via blood to spleen. Recently, the liver sinusoid has been recognized as a special site for blood-lymph translocation of DC by Matsuno et al. [26]. When particulate matters

(e.g. latex) were injected intravenously the administered particles got phagocytosed by immature, transiently phagocytic DC in the liver (or, possibly, DC precursors recruited to the liver) before their translocation through the sinusoid to liver lymphatics and migration to regional hepatic lymph nodes. After intravenous injection of DC, some DC had migrated to the white pulp of spleen as predicted from previous studies, but most were found in hepatic nodes through blood-lymph translocation. Matsuno et al. [26] therefore suggest that the liver sinusoids may act as a biological concentrator of blood DC into hepatic nodes which might be important lymphoid organs, e.g. in transplantation immunity when graft-derived DC reach liver nodes, or in trials using peptide-loaded DC to induce immunity to tumors such as melanoma. An interesting observation made in all studies addressing the migratory properties of DC is that DC arriving in peripheral lymphoid organs successively localize within distinct compartments. Following intravenous administration, for example, DC first bind in the marginal zone of spleen, and then home to the T areas 24 h later to become interdigitating cells [24]. Most of the DC one can isolate from spleen indeed appear derived from DC located in the marginal zone, and besides immigrated DC might comprise immature DC residing in the spleen. Moser and co-workers [27] have recently shown that intravenous injection of LPS induces these marginal zone DC to rapidly (6 h) migrate into the T areas around the central arteriole and to up-regulate CD86 (B7-2). The DC then displayed a phenotype (including expression of the DC-restricted M342 molecule, the significance of which is not yet known) characteristic of the interdigitating cells found in nontreated mice in situ, and of spleen DC matured in vitro during the isolation process. An intriguing finding is that these mature DC have lost their capacity to process antigen but display an increased expression of the DEC-A lectin, DEC-205. The induction of splenic DC maturation in situ is followed by a loss of most DC from spleen resulting in defective antigen presenting function. Thus systemic administration LPS (acting either directly on DC or via induction of inflammatory cytokines) appears to induce a profound loss of DC from nonlymphoid as well as lymphoid tissues. Though puzzling at first it makes sense, therefore, that the same stimuli that mobilize immature DC also recruit a wave of DC progenitors to the same tissues thus providing a feedback mechanism to maintain homeostasis [24]. There is evidence that chemokines might be important in regulating migration of DC and their precursors [28–30].

Mature DC are excellent in stimulating naive T cells as they have retained high levels of MHC peptide complexes generated at the time of contact with the maturation-inducing signal, and during migration and maturation have regulated adhesion (e.g. ICAM-1, LFA-3) as well as costulatory molecules (such as CD86). A recently recogni specialization of mature DC for T-cell sensitization is t release of high levels of biologically active IL-12 upo cell contact. IL-12 production is induced primarily by l tion of CD40 on mature DC by the CD40L expresse activated helper T cells [31, 32], and is likely importar turn on strong cell-mediated immunity.

Induction of Tolerance by Dendritic Cells

There is evidence that DC within thymic medulla critical in inducing clonal deletion to self antigens (cer tolerance). A recent exciting discovery by Süss and Sh man [33] suggests that some of the CD86 expressing within the T areas of spleen express Fas-L (as wel CD8αα and DEC-205) at high levels, and kill CD4+ T c that are responding to antigen by a mechanism that invo Fas-L on the DC and Fas expressed by the activated T c It appears that Fas-L+ DC can also limit proliferatio CD8 T cells by controlling cytokine production by an as undefined, Fas-Fas-L independent mechanism. It is poss that these regulatory Fas-L+ DC normally reside in th areas and tolerize against self antigens, and possibly against immunogenic peptides that are administered temically without adjuvants. Fas-L negative, immigra DC that have captured antigen in the periphery in cont. appear purely stimulatory.

Ontogeny and Differentiation Pathways

The existence and development of distinct functio subsets of DC is an active area of research. It is now est lished that besides the classical immunostimulatory 'm loid-related' there is also a novel type of regulatory 'l phoid-related' DC as exemplified by thymic DC and Fas-L+ regulatory DC subset in spleen. Both DC linea can be generated in vitro from hematopoietic precurs The generation of myeloid DC requires GM-CSF in b mouse and man. In man, myeloid DC can be generated fr early, proliferating CD34+ progenitor cells by GM-CS TNF-α by pathways that are being elucidated [34,35]. S eral subsets of DC develop in such cultures that may cor spond to epithelial DC of the LC type as well as interstit DC and, possibly, to the typical antigen processing DC t have recently been identified in the germinal centers of man tonsil [36]. There is evidence that CLA+ CD34+ c

preferentially develop into DC of the LC type indicating that the spatial organization of the DC system might partially be predetermined at the progenitor level [37]. TGβ$_1$ might be an important regulator of LC ontogeny as TGF-β promotes development of LC in serum-free cultures [38]. Recent data indicate that in man DC can also be generated from nonproliferating, more committed CD14+ progenitors. A substantial fraction of CD14+ cells, i.e. monocyte fraction, in peripheral blood appears to still have the potential to differentiate into macrophages (in the presence of M-CSF) or into DC (in the presence of GM-CSF + IL-4 or IL-13, and TNF-α or monocyte-conditioned medium to induce full maturation) [16–18]. Lymphoid-related DC can also be grown in vitro in mouse and man [39, 40]. Shortman and co-workers [40] have recently shown that GM-CSF is not needed to induce the development of 'lymphoid-derived' DC from early thymic lymphoid precursors but required other cytokines such as IL-1, TNF-α, IL-3, IL-7, SCF and Flt3-L. The DC cultured from the thymic precursors expressed Fas-L like the regulatory CD8+, Fas-L+ DC subset identified in spleen. Recent studies employing GM-CSF or GM-CSF-R-β chain knockout mice quite surprisingly revealed that most of the DC in mouse lymphoid organs are able to develop in the absence GM-CSF or its receptor [41]. At present it is not yet clear whether this means that larger numbers of phenotypically diverse lymphoid-related DC develop, or, alternatively, that myeloid-derived DC can be formed without GM-CSF in vivo. Flt3 ligand is a recently discovered cytokine that turns out to be an effective stimulator of DC development in vivo, and might promote the generation of both myeloid-derived and lymphoid-derived DC [42].

Dendritic Cells in Clinical Immunology

Therapeutic approaches in immune-mediated diseases have so far concentrated on antigens and T cells. Given the exceptional role of DC in cell-mediated immunity it may be rewarding to investigate and manipulate immune responses at the level of antigen presenting cells, particularly DC. A recent example is HIV-1 infection where DC appear to contribute in a major way. HIV-1 readily enters immature DC but productive interaction only occurs in DC-T cell conjugates [43]. HIV-1 causes DC and memory T cells to fuse, and the resulting heterokaryons or syncytia are sites of vigorous viral replication and cell death in vitro [43] and in vivo [44]. Given the exceptional capacity to induce immunity in vivo, researchers are now trying to use antigen-laden DC for boosting host resistance in vivo, notably to tumors. A very first clinical trial successfully vaccinated patients with B-cell lymphoma using autologous antigen-pulsed DC directly isolated from blood [45]. Several groups are now trying to use DC expanded in culture as adjuvants for enhancing immune responses to tumors such as melanoma, as animal experiments have been promising [46]. The recent identification of down-regulatory DC subsets, and their generation in culture may allow a novel approach to induce tolerance in transplantation, autoimmunity, and allergy. Molecular biology approaches have already identified several novel DC-specific molecules such as DEC-205 [23], and will hopefully also deepen our understanding of the transcriptional regulation of DC maturation that is currently restricted to an ill-defined role of relB [47]. Such knowledge might allow us then to manipulate DC selectively in vitro as well as in vivo.

References

1 Stingl G, Bergstresser PR: Dendritic cells: A major story unfolds. Immunol Today 1995;16:330–333.

2 Steinman RM, Cohn ZA: Identification of a novel cell type in peripheral lymphoid organs of mice. I. Morphology, quantitation, tissue distribution. J Exp Med 1973;137:1142–1162.

3 Schuler G, Steinman RM: Murine epidermal Langerhans cells mature into potent immunostimulatory dendritic cells in vitro. J Exp Med 1985;161:526–546.

4 Romani N, Koide S, Crowley M, Witmer-Pack M, Livingstone AM, Fathman CG, Inaba K, Steinman RM: Presentation of exogenous protein antigens by dendritic cells to T cell clones: Intact protein is presented best by immature epidermal Langerhans' cells. J Exp Med 1989;169:1169–1178.

5 Stössel H, Koch F, Kämpgen E, Stöger P, Lenz A, Heufler C, Romani N, Schuler G: Disappearance of certain acidic organelles (endosomes and Langerhans' cell granules) accompanies loss of antigen processing capacity upon culture of epidermal Langerhans' cells. J Exp Med 1990;172:1471–1482.

6 Kämpgen E, Koch N, Koch F, Stöger P, Heufler C, Schuler G, Romani N: Class II major histocompatibility complex molecules of murine dendritic cells: Synthesis, sialylation of invariant chain, and antigen processing capacity are down-regulated upon culture. Proc Natl Acad Sci USA 1991;88:3014–3018.

7 Heufler C, Koch F, Schuler G: Granulocyte-macrophage colony-stimulating factor and interleukin-1 mediate the maturation of murine epidermal Langerhans' cells into potent immunostimulatory dendritic cells. J Exp Med 1988;167:700–705.

8 Larsen CP, Steinman RM, Witmer-Pack M, Hankins DF, Morris PJ, Austyn JM: Migration and maturation of Langerhans' cells in skin transplants and explants. J Exp Med 1990;172:1483–1493.

9 Holt PG, Oliver J, Bilyk N, McMenamin C, McMenamin PG, Kraal G, Thepen T: Downregulation of the antigen presenting cell function(s) of pulmonary dendritic cells in vivo by resident alveolar macrophages. J Exp Med 1993;177:397–407.

10 Austyn JM, Hankins DF, Larsen CP, Morris PJ, Rao AS, Roake JA: Isolation and characterization of dendritic cells from mouse heart and kidney. J Immunol 1994;152:2401–2410.
11 O'Doherty U, Steinman RM, Peng M, Cameron PU, Gezelter S, Kopeloff I, Swiggard WJ, Pope M, Bhardwaj N: Dendritic cells freshly isolated from human blood express CD4 and mature into typical immunostimulatory dendritic cells after culture in monocyte-conditioned medium. J Exp Med 1993;178:1067–1078.
12 Steinman RM: The dendritic cell system and its role in immunogenicity. Annu Rev Immunol 1991;9:271–296.
13 Caux C, Dezutter-Dambuyant C, Schmitt D, Banchereau J: GM-CSF and TNF-α cooperate in the generation of dendritic Langerhans' cells. Nature 1992;360:258–261.
14 Inaba K, Steinman RM, Pack MW, Aya H, Inaba M, Sudo T, Wolpe S, Schuler G: Identification of proliferating dendritic cell precursors in mouse blood. J Exp Med 1992;175:1157–1167.
15 Strunk D, Rappersberger K, Egger C, Strobl H, Krömer E, Elbe A, Maurer D, Stingl G: Generation of human dendritic cells Langerhans' cells from circulating CD34$^+$ hematopoietic progenitor cells. Blood 1996;87:1292–1302.
16 Sallusto F, Lanzavecchia A: Efficient presentation of soluble antigen by cultured human dendritic cells is maintained by granulocyte/macrophage colony-stimulating factor plus interleukin-4 and downregulated by tumor necrosis factor α. J Exp Med 1994;179:1109–1118.
17 Bender A, Sapp M, Schuler G, Steinman RM, Bhardwaj N: Improved methods for the generation of dendritic cells from nonproliferating progenitors in human blood. J Immunol Methods 1996;196:121–135.
18 Romani N, Reider D, Heuer M, Ebner S, Kämpgen E, Eibl B, Niederwieser D, Schuler G: Generation of mature dendritic cells from human blood – An improved method with special regard to clinical applicability. J Immunol Methods 1996;196:137–151.
19 Zhou L-J, Tedder TF: Human blood dendritic cells selectively express CD83, a member of the immunoglobulin superfamily. J Immunol 1995; 154:3821–3835.
20 Lanzavecchia A: Mechanisms of antigen uptake for presentation. Curr Opin Immunol 1996;8: 348–354.
21 Sallusto F, Cella M, Danieli C, Lanzavecchia A: Dendritic cells use macropinocytosis and the mannose receptor to concentrate macromolecules in the major histocompatibility complex class II compartment: Downregulation by cytokines and bacterial products. J Exp Med 1995; 182:389–400.
22 Fiebiger E, Stingl G, Maurer D: Anti-IgE and anti-Fc epsilonRI autoantibodies in clinical allergy. Curr Opin Immunol 1996;8:784–789.
23 Jiang W, Swiggard WJ, Heufler C, Peng M, Mirza A, Steinman RM, Nussenzweig MC: The receptor DEC-205 expressed by dendritic cells and thymic epithelial cells is involved in antigen processing. Nature 1995;375:151–155.
24 Austyn JM: New insights into the mobilization and phagocytic activity of dendritic cells. J Exp Med 1996;183:1287–1292.
25 Kleijmeer MJ, Oorschot VMJ, Geuze HJ: Human resident Langerhans' cells display a lysosomal compartment enriched in MHC class II. J Invest Dermatol 1994;103:516–523.
26 Matsuno K, Ezaki S, Kudo S, Uehara Y: A life stage of particle-laden rat dendritic cells in vivo: Their terminal division, active phagocytosis, and translocation from the liver to hepatic lymph. J Exp Med 1996;183:1865–1878.
27 De Smedt T, Pajak B, Muraille E, Heinen E, De Baetselier P, Urbain J, Leo O, Moser M: Positive and negative regulation of dendritic cell function by lipopolysaccharide in vivo. J Exp Med 1996;184:1413–1424.
28 Nakamura K, Williams IR, Kupper TS: Keratinocyte-derived monocyte chemoattractant protein 1 (MCP-1): Analysis in a transgenic model demonstrates MCP-1 can recruit dendritic and Langerhans' cells to skin. J Invest Dermatol 1995;105:635–643.
29 Sozzani S, Sallusto F, Luini W, Zhou D, Piemonti L, Allavena P, van Damme J, Valitutti S, Lanzavecchia A, Mantovani A: Migration of dendritic cells in response to formyl peptides, C5a, and a distinct set of chemokines. J Immunol 1995;155:3292–3295.
30 Xu LL, Warren MK, Rose WL, Gong WH, Wang JM: Human recombinant monocyte chemotactic protein and other c-c chemokines bind and induce directional migration of dendritic cells in vitro. J Leukoc Biol 1996;60:365–371.
31 Cella M, Scheidegger D, Palmer-Lehmann K, Lane P, Lanzavecchia A, Alber G: Ligation of CD40 on dendritic cells triggers production of high levels of interleukin-12 and enhances T cell stimulatory capacity: T-T help via APC activation. J Exp Med 1996;184:747–752.
32 Koch F, Stanzl U, Jennewein P, Janke K, Heufler C, Kämpgen E, Romani N, Schuler G: High level IL-12 production by murine dendritic cells: Upregulation via MHC class II and CD40 molecules and downregulation by IL-4 and IL-10. J Exp Med 1996;184:741–746.
33 Süss G, Shortman K: A subclass of dendritic cells kills CD4 T cells via Fas/Fas-ligand-induced apoptosis. J Exp Med 1996;183:1789–1796.
34 Caux C, Vanbervliet B, Massacrier C, Dezutter-Dambuyant C, De Saint-Vis B, Jacquet C, Yoneda K, Imamura S, Schmitt D, Banchereau J: CD34$^+$ hematopoietic progenitors from human cord blood differentiate along two independent dendritic cell pathways in response to GM-CSF+TNFα. J Exp Med 1996;184:695–706.
35 Szabolcs P, Avigan D, Gezelter S, Ciocon DH, Moore MAS, Steinman RM, Young JW: Dendritic cells and macrophages can mature independently from a human bone marrow-derived, post-colony-forming unit intermediate. Blood 1996;87:4520–4530.
36 Liu YJ, Grouard G, de Bouteiller O, Banchereau J: Follicular dendritic cells and germinal centers. Int Rev Cytol 1996;166:139–179.
37 Strunk D, Egger C, Leitner G, Stingl G: A skin homing molecule defines the circulating progenitor of epidermal dendritic cells. Submitted.
38 Strobl H, Riedl E, Scheinecker C, Bello-Fernandez C, Pickl WF, Rappersberger K, Majdic O, Knapp W: TGF-β$_1$ promotes in vitro development of dendritic cells from CD34$^+$ hemopoietic progenitors. J Immunol 1996;157: 1499–1507.
39 Res P, Martinez-Cáceres E, Jaleco AC, Staal F, Noteboom E, Weijer K, Spits H: CD34$^+$ CD38^{dim} cells in the human thymus can differentiate into T, natural killer, and dendritic cells but are distinct from pluripotent stem cells. Blood 1996;87:5196–5206.
40 Saunders D, Lucas K, Ismaili J, Wu L, Maraskovsky E, Dunn A, Shortman K: Dendritic cell development in culture from thymic precursor cells in the absence of granulocyte-macrophage colony stimulating factor. J Exp Med 1997, in press.
41 Vremec D, Lieschke GJ, Dunn AR, Robb L, Metcalf D, Shortman K: The influence of granulocyte-macrophage colony-stimulating factor on dendritic cell levels in mouse lymphoid organs. Eur J Immunol 1997, in press.
42 Maraskovsky E, Brasel K, Teepe M, Roux ER, Lyman SD, Shortman K, McKenna HJ: Dramatic increase in the number of functionally mature dendritic cells in Flt3 ligand-treated mice: Multiple dendritic cell subpopulations identified. J Exp Med 1997, in press.
43 Pope M, Gezelter S, Gallo N, Hoffman L, Steinman RM: Low level of HIV-1 infection in cutaneous dendritic cells promote extensive viral replication upon binding to memory CD4$^+$ T cells. J Exp Med 1995;182:2045–2056.
44 Frankel SS, Wenig BM, Burke AP, Mannan P, Thompson LDR, Abbondanzo SL, Nelson AM, Pope M, Steinman RM: Replication of HIV-1 in dendritic cell-derived syncytia at the mucosal surface of the adenoid. Science 1996;272:115–117.
45 Hsu FJ, Benike C, Fagnoni F, Liles TM, Czerwinski D, Taidi B, Engleman EG, Levy R: Vaccination of patients with B-cell lymphoma using autologous antigen-pulsed dendritic cells. Nature Med 1996;2:52–58.
46 Young JW, Inaba K: Dendritic cells as adjuvants for class I major histocompatibility complex-restricted antitumor immunity. J Exp Med 1996;183:7–11.
47 Burkly L, Hession C, Ogata L, Reilly C, Marconi LA, Olson D, Tizard R, Cate R, Lo D: Expression of *relB* is required for the development of thymic medulla and dendritic cells. Nature 1995;373:531–536.

experienced an "extra" duplication event. This suggests that a fish-specific *HOX* cluster duplication occurred before the divergence of *Fugu* and zebrafish lineages more than 150 million years ago (*15*), but after the divergence of ray-finned and lobe-finned lineages. Goldfish, salmonids, and some other teleosts have experienced additional, more recent polyploidization events (*16*). Genomic analysis of basally branching ray-finned fish, such as sturgeons, *Amia*, or *Polypterus*, is necessary to clarify the timing of the *HOX* duplication event.

To determine whether "extra" fish *hox* clusters result from tandem duplication or chromosome duplication in fish, or cluster loss in tetrapods, we mapped zebrafish *hox* clusters: cloned, sequenced, and mapped four new genes whose orthologs are syntenic with *HOX* clusters in mammals (*dhh*, *evx1*, *en2b*, and *gli3*), and mapped four previously unmapped zebrafish genes [*dlx5*, *dlx6*, *dlx8*, and *pl10a*; see (*11*)] whose orthologs are linked to *HOX* clusters in mammals. These experiments showed that zebrafish has two copies of each *HOX* chromosome segment in mammals (Fig. 4). For example, the human and mouse *HOXB* chromosomes have six and four genes, respectively, whose apparent orthologs map on one of the two zebrafish chromosomes containing *hoxba* or *hoxbb* (Fig. 4). Each of these two chromosomes also has one copy of other duplicate genes, including *dlx7/dlx8*, *rara2a/rara2b*, and *hbae4/hbae1* (*11*, *17*). We conclude that zebrafish has two copies of this mammalian chromosome segment. Because similar results were obtained for the other clusters (Fig. 4), we infer that *hox* cluster duplication in ray-finned fish occurred by whole chromosome duplication. Although we found a single *hoxd* cluster in zebrafish, mapping experiments identified the predicted duplicate chromosome segments (Fig. 4), suggesting secondary loss of one *hoxd* duplicate.

These results suggest two rounds of *HOX* chromosome duplication (probably whole genome duplication) before the divergence of ray-finned and lobe-finned fishes, and one more in ray-finned fish before the teleost radiation. Because gene duplicates often have a subset of the functions of the ancestral gene (*18*), mutations in duplicate genes may reveal essential functions that otherwise might remain hidden. For example, if a gene is essential for distinct early and late functions, a lethal mutation knocking out the early function might obscure the late function in a mutant mammal, but both functions would be evident if the two functions assort to different zebrafish gene duplicates. The conclusion that the genetic complexity of *hox* clusters in teleost fish has exceeded that of mammals for more than 100 million years calls into question the concept of a tight linkage of *HOX* cluster number and morphological complexity along the body axis. However, because teleosts are the most species-rich group of vertebrates and exhibit tremendous morphological diversity, it is tempting to speculate that the duplication event detected here may have provided gene copies that helped spur the teleost radiation.

References and Notes

1. R. Krumlauf, *Cell* **78**, 191 (1994); M. Kmita-Cunisse, F. Loosli, J. Bierne, W. Gehring, *Proc. Natl. Acad. Sci. U.S.A.* **95**, 3030 (1998); M. Kourakis *et al.*, *Dev. Biol.* **190**, 284 (1997); E. B. Lewis, *Nature* **276**, 565 (1978); S. Dickman, *Science* **275**, 1568 (1997).
2. D. Duboule and P. Dollé, *EMBO J.* **8**, 1497 (1989).
3. J. Garcia-Fernandez and P. W. H. Holland, *Nature* **370**, 563 (1994); A. Di Gregorio *et al.*, *Gene* **156**, 253 (1995).
4. P. W. H. Holland and J. Garcia-Fernandez, *Dev. Biol.* **173**, 382 (1996); A. Sharman and P. W. H. Holland, *Neth. J. Zool.* **46**, 47 (1996).
5. S. Aparicio *et al.*, *Nature Genet.* **16**, 79 (1997).
6. B. Misof and G. Wagner, *Mol. Phylogenet. Evol.* **5**, 309 (1996).
7. F. van der Hoeven, P. Sordino, N. Fraudeau, J. C. Izpisua-Belmonte, D. Duboule, *Mech. Dev.* **54**, 9 (1996); B. Misof, M. Blance, G. Wagner, *J. Exp. Zool.* **274**, 193 (1996); V. Prince, L. Joly, M. Ekker, R. Ho, *Development* **125**, 407 (1998).
8. H. G. Eiken, P. R. Njølstad, A. Molven, A. Fjose, *Biochem. Biophys. Res. Commun.* **149**, 1165 (1987); P. Njølstad, A. Molven, I. Hordvik, J. Apold, A. Fjose, *Nucleic Acids Res.* **16**, 9097 (1988); P. R. Njølstad, A. Molven, H. G. Eiken, A. Fjose, *Gene* **73**, 33 (1989); P. R. Njølstad, A. Molven, J. Apold, A. Fjose, *EMBO J.* **9**, 515 (1990); J. A. Runstadler and T. D. Kocher, *Nucleic Acids Res.* **19**, 5434 (1991); P. Sordino, D. Duboule, T. Kondo, *Mech. Dev.* **59**, 165 (1996); V. Prince, C. Moens, C. Kimmel, R. Ho, *Development* **125**, 393 (1997).
9. DNA pools (plate pools, then row and column pools) from an arrayed zebrafish PAC library (*19*) were screened with redundant primers (Hox-F: 5′-CGNAA-GAARMGNGTNCCNTAYAC-3′; Hox-R: 5′-CATNCKN-CKRTTYTGRAACCANAT-3′; Hox-Fi: 5′-CTNGARYT-NGARAARGARTTYCA), where N is A, C, T, or G, R is A or G, M is A or C, Y is C or T, and K is T or G. Ends of positive PACs were sequenced and specific primers used to find overlapping clones. Positive PACs were amplified with redundant primers; products were cloned and sequenced, and gene specific primers were used to obtain sequence directly from PAC DNA.
10. Unambiguously alignable sequences were obtained using CLUSTAL X (http://www-igbmc.u-strasbg.fr/BioInfo/ClustalX/Top.html) and trees were generated by the neighbor-joining method [N. Saitou and M. Nei, *Mol. Biol. Evol.* **4**, 406 (1987)]. A lamprey (*Petromyzon marinus*) cDNA library screened with redundant *hox* gene primers provided an outgroup. For accession numbers, see (*11*).
11. See *Science* Online (www.sciencemag.org) for a catalog of zebrafish *hox* genes, alignments for the phylogenetic trees in Fig. 2, accession numbers of loci used to construct the phylogenetic trees, mapping primers for zebrafish genes, and mapping data for newly mapped loci.
12. P. Ahlberg and A. Milner, *Nature* **368**, 507 (1994).
13. J. Zhang and M. Nei, *Genetics* **142**, 295 (1996); C. Kappen and F. Ruddle, *Curr. Opin. Genet. Dev.* **3**, 931 (1993).
14. W. Bailey, J. Kim, G. Wagner, F. Ruddle, *Mol. Biol. Evol.* **14**, 843 (1997); A. Meyer, *Nature* **391**, 225 (1998).
15. P. Cantatore *et al.*, *J. Mol. Evol.* **39**, 589 (1994).
16. D. C. Buth, T. Dowling, J. R. Gold, in *Cyprinid Fishes: Systematics, Biology, and Exploitation*, I. J. Winfield and J. S. Nelson, Eds. (Chapman and Hall, London, 1991), pp. 83–126; F. W. Allendorf and G. H. Thorgaard, in *Evolutionary Genetics of Fishes*, J. B. Turner, Ed. (Plenum, New York, 1984), pp. 1–53.
17. J. H. Postlethwait *et al.*, *Nature Genet.* **18**, 345 (1998).
18. S. D. Ferris and G. S. Whitt, *J. Mol. Evol.* **12**, 267 (1979); A. L. Hughes, *Proc. R. Soc. London Ser. B* **256**, 119 (1994); A. Force *et al.*, in preparation.
19. C. Amemiya, unpublished data.

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Regulation of the Proinflammatory Effects of Fas Ligand (CD95L)

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Fas ligand (CD95L) inhibits T cell function in immune-privileged organs such as the eye and testis, yet in most tissues CD95L expression induces potent inflammatory responses. With a stably transfected colon carcinoma cell line, CT26-CD95L, the molecular basis for these divergent responses was defined. When injected subcutaneously, rejection of CT26-CD95L was caused by neutrophils activated by CD95L. CT26-CD95L survived in the intraocular space because of the presence of transforming growth factor–β (TGF-β), which inhibited neutrophil activation. Providing TGF-β to subcutaneous sites protected against tumor rejection. Thus, these cytokines together generate a microenvironment that promotes immunologic tolerance, which may aid in the amelioration of allograft rejection.

The CD95 protein (also called Fas or APO-1) is a cell surface receptor that activates the death signaling pathway in cells. Its physiological ligand, CD95L, can transduce this signal upon cell contact (*1*). The CD95-CD95L system has been implicated in the clonal deletion of autoreactive lymphocytes in peripheral lymphoid tissues and in the elimination of autoreactive lymphocyte populations (*2*), thus contributing to homeostasis

of the immune system. CD95L expression in normal tissue is restricted to T lymphocytes, macrophages, the cornea, the iris, ciliary bodies, the retina, and Sertoli cells (*3*). Through its ability to suppress both cellular and humoral immunity (*2*, *4*), CD95L has been implicated in maintenance of the immune-privileged status in the eye (*3*) and testis (*5*). CD95L may also confer immune suppression in malignancy (*6*) or be useful in delaying rejection of allogeneic cells (*4*, *7*) by promoting immune evasion.

However, CD95L is also proinflammatory (*8*, *9*). Expression of CD95L on myotubes or pancreatic islets of transgenic mice induces a granulocytic response that accelerates graft rejection (*10*). Differences in the effect of CD95L at distinct anatomic sites may be caused by secondary factors that modulate its function (*11*). Here we attempt to identify factors that could explain the paradoxical effects of CD95L in immune-privileged sites and immunocompetent tissues.

To determine whether CD95L could stimulate an inflammatory response in an immune-privileged tissue, we injected 10^5 CT26-CD95L cells (*4*) (n = 5 mice) or a CD95L-negative control cell line, CT26-Neo (n = 4 mice), into the anterior chamber of the eye of syngeneic Balb/c mice. Both cell lines produced tumors by 8 days in all mice at the intraocular site. In contrast, tumors grew only from CT26-Neo cells when the tumor cells were injected subcutaneously (*12*). Thus, the microenvironment, rather than the amount of CD95L, determined the ability of these cells to induce inflammatory responses that inhibited tumor growth.

Rejection of CD95L$^-$ tumors occurs in *scid-beige* mice and is thus independent of T cell and natural killer (NK) cell function (*8*). Polymorphonuclear leukocytes (PMNs) infil-

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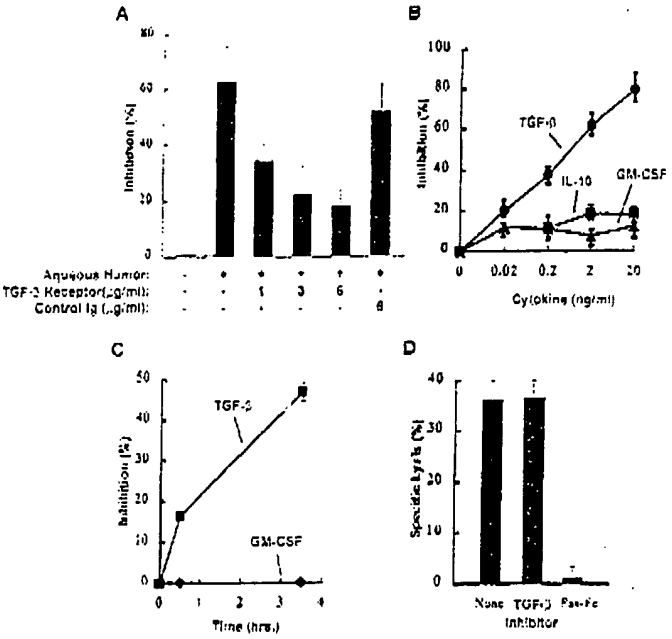

Fig. 2. Inhibitory effects of recombinant TGF-β on neutrophil-mediated cytotoxicity and its role in the aqueous humor. (**A**) Inhibitory effects of aqueous humor and reversal by neutralizing soluble TGF-β receptor protein. Mouse neutrophils were incubated with radiolabeled CT26-CD95L at a ratio of 50:1 and 40 μl of bovine aqueous humor, obtained immediately after the animal was killed and stored at −70°C. The indicated concentrations of human TGF-β–soluble receptor-Fc fusion protein (TGF-β SRII/Fc; R&D Systems) or control human immunoglobulin were added to the assay as shown in Fig. 1C. The specific lysis without inhibitor was 44.3%. (**B**) Inhibition of neutrophil cytolysis by TGF-β1. Human PMNs were incubated with CT26-CD95L cells at an E/T ratio of 100:1. Increasing amounts of human TGF-β1 (R&D Systems), human interleukin-10 (Genzyme), and granulocyte-macrophage colony-stimulating factor (GM-CSF; Immunex) were added to the culture, and Cr release assays were performed as in Fig. 1. The specific lysis without inhibitors was 44.3%. (**C**) Inhibition of neutrophil-mediated cytolysis of CT26-CD95L cells by preincubation of neutrophils with TGF-β1. Human PMNs were preincubated with TGF-β1 (20 ng/ml) or human GM-CSF (20 ng/ml) for the indicated period and washed with 10 ml of medium three times. The percent inhibition was calculated relative to neutrophils preincubated with medium, TGF-β1, or GM-CSF for the same period of time. The data represent the mean ± SE from three independent experiments. (**D**) TGF-β1 does not inhibit CD95L-mediated apoptosis in Jurkat cells. CT26-CD95L cells were incubated with ^{51}Cr-labeled Jurkat cells (1×10^4 per well) at a ratio of 10:1. Human TGF-β1 (20 ng/ml) was added to the medium, and CD95-Fc fusion protein was used as a positive control. Cytotoxicity was measured by ^{51}Cr release after 4 hours of coincubation. The data represent the mean ± SD from three independent experiments.

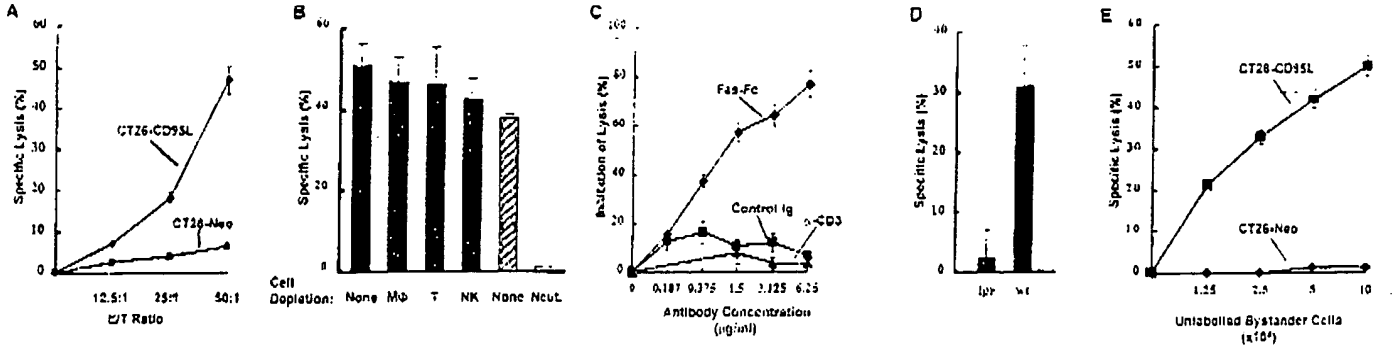

Fig. 1. PMN-mediated destruction of CD95L$^+$ CT26 cells but not CD95L$^-$ CT26 cells in vitro and CD95L involvement in neutrophil cytolytic function. (**A**) Dose-dependent cytolysis of CD95L$^+$ CT26 cells by human neutrophils. Human neutrophils (*20*) of ≥97% purity were incubated with ^{51}Cr-labeled CT26-CD95L or CT26-Neo cells for 19 hours on fibronectin-coated plates (*21*) at the indicated ratios. The data represent the mean ± SE of three independent experiments. (**B**) PMN-mediated cytolysis. Human PMNs (black bars) were depleted of T lymphocytes, NK cells, or macrophages by immunomagnetic bead separation (*22*) with antibodies to CD3, CD65, or CD115/c (Neomarkers: Pharmingen) and were mixed with CD95L$^+$ cells (50:1 ratio). Mouse neutrophils (gray bars) (*23*) were mixed with CD95L$^-$ cells (50:1 ratio). Mouse spleen cells depleted of neutrophils with antibody to Ly6G (Pharmingen) (*22*) were used as a negative control. (**C**) Inhibition of neutrophil cytotoxicity by CD95-Fc (*4*). Radiolabeled CT26-CD95L cells were mixed with neutrophils [effector/target (E/T) ratio, 50:1], and mouse CD95-Fc protein, control human immunoglobulin, or antibody to CD3 (OKT3, Ortho Biotech) was added. The specific lysis without inhibitor was 37.5%. (**D**) Lysis of CT26-CD95L by neutrophils from *lpr*$^-$/*lpr*$^-$ or wild-type congenic C57BL/6 mice at an E/T ratio of 25:1. (**E**) Induction of bystander cytotoxicity by CT26-CD95L cells. CT26-Neo target cells were labeled with ^{51}Cr and mixed with neutrophils at an E/T ratio of 100:1 in the presence of the indicated numbers of unlabeled CT26-CD95L cells. Equal numbers of unlabeled CT26-Neo cells were used as a negative control.

trate CD95L tumors in the skin and contribute to the CD95L antitumor response (*8, 9*), but the mechanism by which these cells promote tumor rejection is unknown. We therefore determined whether neutrophils could directly lyse CT26-CD95L cells in vitro. Human PMNs, incubated with CT26-CD95L or CT26-Neo target cells, lysed only CT26-CD95L (Fig. 1A). Depletion of the effector population with antibodies to T cells, NK cells, or macrophages did not reduce this cytolysis. PMNs derived from peripheral blood leukocytes of syngeneic Balb/c mice lysed CT26-CD95L tumor cells, in contrast to a control cell population depleted with a neutrophil-specific antibody, which did not retain this activity (Fig. 1B). Thus, PMNs interacted directly with CD95L$^+$ cells to mediate their destruction.

Lysis of CT26-CD95L cells was inhibited specifically by a CD95-Fc fusion protein but not by a negative control immunoglobulin (Fig. 1C) and was markedly reduced in neutrophils from *lpr* mice (Fig. 1D), which express a defective CD95 receptor. Bystander cells that did not express CD95L were lysed when chromium-labeled CT26-neo cells were incubated with unlabeled CT26-CD95L cells (Fig. 1E), which suggests that lysis of CT26-CD95L cells was not due to their intrinsic susceptibility to lysis but instead to the ability of CD95L to induce neutrophil cytotoxicity locally.

Tumors can grow intraocularly but not in subcutaneous sites. The microenvironment of the eye may contain factors, therefore, that suppress PMN activation. We tested the fluid of the anterior chamber (the aqueous humor) in cytotoxicity assays and found that it inhibited CD95L activation of PMN lytic activity (Fig. 2A). We tested several cytokines known to be present in the aqueous humor (*13*). Transforming growth factor-β1 (TGF-β1) inhibited PMN cytotoxicity in vitro (Fig. 2B), and a soluble TGF-β receptor-Fc fusion protein inhibited the suppressive effect of aqueous humor in vitro (Fig. 2A). The effect of TGF-β was on the PMNs (Fig. 2C), and the same dose of TGF-β1 had no effect on CD95L-dependent apoptosis of Jurkat cells (Fig. 2D). A similar inhibitory effect on neutrophil function was observed with human TGF-β2 (*14*).

To understand the mechanism of PMN inhibition further, we examined the activity of mitogen-activated protein kinase (MAPK) in CD95-stimulated human neutrophils. Rapid activation of p38 MAPK activity, as determined on its substrate, ATF-2 (*15*), was demonstrated in CD95L-stimulated neutrophils (Fig. 3A, lane 2). Preincubation of neutrophils with TGF-β1 suppressed this CD95L-induced activation of p38 MAPK (Fig. 3A, lane 3). Similarly to TGF-β, incubation of neutrophils with the p38 MAPK inhibitors SB203580 and SB202190 reduced neutrophil-mediated cytolysis, in contrast to a p44/42 antagonist, PD98059, that had no effect (Fig. 3B). These results demonstrated that CD95L-induced neutrophil cytotoxicity was dependent on p38 MAPK function, which is inhibited by TGF-β.

To determine whether expression of TGF-β could affect the proinflammatory effect of CD95L in subcutaneous tissue, CT26-CD95L cells were stably transfected with an expression vector encoding a constitutively active form of TGF-β1. Histologic analysis confirmed fibrosis and regression of CT26-CD95L, as reported previously (*8*), in contrast to the robust tumor growth of the double transfectants (Fig. 4A, upper panel). Occasional neutrophils were observed in the TGF-β–expressing tumors and in intraocular CT26-CD95L (Fig. 4A, lower panel), which suggests that TGF-β suppressed CD95L-induced PMN activation, although an effect on migration or survival in vivo cannot be excluded. All CT26-CD95L/TGF-β cells (n = 8) grew in recipient mice when inoculated subcutaneously, in contrast to no growth in any recipient of CT26-CD95L cells (n = 8) (Fig. 4B).

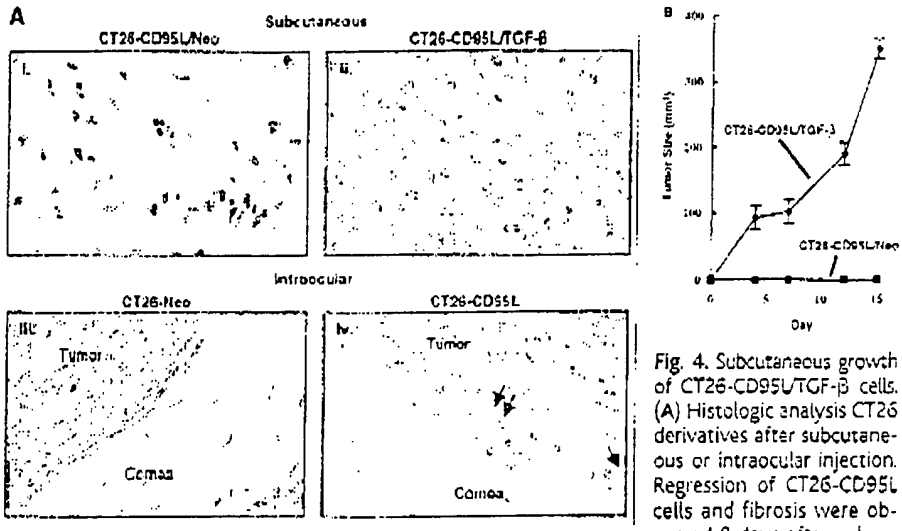

Fig. 4. Subcutaneous growth of CT26-CD95L/TGF-β cells. (A) Histologic analysis CT26 derivatives after subcutaneous or intraocular injection. Regression of CT26-CD95L cells and fibrosis were observed 8 days after subcutaneous inoculation (i), in contrast to the observation of viable tumor cells after injection with CT26-CD95L/TGF-β cells (ii). Tumor growth was observed intraocularly for both CT26-Neo (iii) and CT26-CD95L (iv) tumors. Arrows indicate the presence of occasional neutrophils in CT26-CD95L tumors intraocularly (iv). (B) Mice were injected subcutaneously with CT26-CD95L cells (n = 8) or with a subline stably transduced with a retroviral vector encoding constitutively active TGF-β1 (n = 8). Tumor volume, measured with calipers, was calculated from the largest (a) and smallest (b) diameter with the formula $0.5a \times b^2$. CT26-CD95L/TGF-β cells were generated by infection in CT26-CD95L cell line with a retrovirus vector (*24*). A constitutively active human TGF-β1 gene (*25*) was cloned into retroviral vector LZRS-pBMN-LacZ (*26*) by replacing the *lacZ* gene. TGF-β1 levels in supernatants 48 hours after infection were 8 ng/ml of TGF-β1, determined by enzyme-linked immunosorbent assay (R&D Systems). CT26-CD95L cells infected with control retroviruses (CT26-CD95L/Neo) were used as controls.

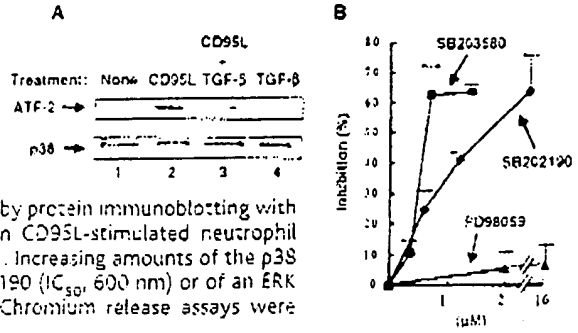

Fig. 3. p38 MAPK activation by CD95L, inhibition by TGF-β, and abrogation of CD95L-induced neutrophil cytolysis by p38 MAPK antagonists. (A) Activation and modulation of p38 MAPK in neutrophils by CD95L and inhibition by TGF-β. (Upper panel) Cellular p38 MAPK activity was determined by phosphorylation of an ATF2 substrate (New England Biolabs) after immunoprecipitation. Neutrophils were pretreated with human TGF-β1 (20 ng/ml, lanes 3 and 4) or with medium (lanes 1 and 2) at 4°C for 1 hour. Subsequently, the neutrophils were cocultured with human CD95L (400 ng/ml) (Upstate Biotechnology; lanes 2 and 3) or with medium (lanes 1 and 4) for 10 min at 37°C. (Lower panel) The total amount of p38 MAPK was examined by protein immunoblotting with p38 kinase antibody (New England Biolabs). (B) Effects of p38 MAPK inhibitors on CD95L-stimulated neutrophil cytotoxicity. Neutrophils were incubated with CT26-CD95L cells at an E/T ratio of 50:1. Increasing amounts of the p38 MAPK inhibitors SB203580 [median inhibitory concentration (IC_{50}), 350 nm] or SB202190 (IC_{50}, 600 nm) or of an ERK kinase inhibitor (negative control), PD988059 (IC_{50}, 2 μm), were added to media. Chromium release assays were performed as in Fig. 2. The specific lysis without inhibitor was 45%.

The proinflammatory effects of CD95L have raised questions about its contribution to immune privilege (*3, 5, 16*), tolerance, and graft survival (*9, 10*). Although it triggers apoptosis in T lymphocytes (*2*) (Fig. 2D), CD95L unexpectedly stimulated PMN activation. As described for other PMN stimulants (*17*), this activity is dependent on its ability to enhance p38 MAPK activity (Fig. 3). PMNs directly mediate cytolysis of CD95L$^+$ cells, and this effect is inhibited by TGF-β, which is present in the aqueous humor (*13*). TGF-β also plays a role in immune tolerance through this mechanism and its effect on T cell proliferation. Although it inhibits p38 MAPK activity in other cells (*18*), its effect on innate immune responses mediated by neutrophils was previously unknown. Together CD95L and TGF-β promote lymphocyte clonal deletion and suppress inflammation. Thus, providing a microenvironment that includes both of these elements may aid in amelioration of allograft rejection at nonprivileged sites. Both CD95L and TGF-β1 have also been detected in tumors, particularly in the extracellular matrix, where they may inhibit immunologic recognition of malignancies (*6, 19*). Successful immune therapies for cancer are likely to require strategies to reverse this mechanism of immune suppression in vivo.

References and Notes

1. N. Itoh *et al.*, *Cell* **66**, 233 (1991); T. Suda, T. Takahashi, P. Golstein, S. Nagata, *ibid.* **75**, 1169 (1993).
2. T. Brunner *et al.*, *Nature* **373**, 441 (1995); J. Dhein, H. Walczak, C. Baumler, K.-M. Debatin, P. H. Krammer, *ibid.*, p. 438; S. Ju *et al.*, *ibid.*, p. 444, S. Nagata and P. Golstein, *Science* **267**, 1449 (1995).
3. T. S. Griffith, T. Brunner, S. M. Fletcher, D. R. Green, T. A. Ferguson, *Science* **270**, 1189 (1995).
4. H. Arai, S. Y. Chan, D. K. Bishop, C. J. Nabel, *Nature Med.* **3**, 843 (1997).
5. D. Bellgrau *et al.*, *Nature* **377**, 630 (1995).
6. J. O'Connell, G. C. O'Sullivan, J. K. Collins, F. Shanahan, *J. Exp. Med.* **184**, 1075 (1996); M. Hahne *et al.*, *Science* **274**, 1363 (1996); S. Strand *et al.*, *Nature Med.* **2**, 1361 (1996).
7. H. T. Lau, M. Yu, A. Fontana, C. J. Stoeckert Jr., *Science* **273**, 109 (1996).
8. H. Arai, D. Gordon, E. G. Nabel, G. J. Nabel, *Proc. Natl. Acad. Sci. U.S.A.* **94**, 13862 (1997).
9. K.-i. Seino, N. Kayagaki, K. Okumura, H. Yagita, *Nature Med.* **3**, 165 (1997).
10. J. Allison, H. M. Georgiou, A. Strasser, D. L. Vaux, *Proc. Natl. Acad. Sci. U.S.A.* **94**, 3943 (1997); A. V. Chervonsky *et al.*, *Cell* **89**, 17 (1997); S.-M. Kang *et al.*, *Nature Med.* **3**, 738 (1997); S.-M. Kang *et al.*, *Science* **278**, 1322 (1997).
11. D. R. Green and C. F. Ware, *Proc. Natl. Acad. Sci. U.S.A.* **94**, 5986 (1997).
12. Eight-week-old female BALB/c mice from Jackson Laboratory were injected with CT26-CD95L or CT26-neo cells (10^5) in the right anterior chamber of the eye or subcutaneously into the flank after cells were harvested with trypsin, incubated in growth medium at 37°C for 1 hour, washed with phosphate-buffered saline, and resuspended in saline.
13. J. W. Streilein, G. A. Wilbanks, A. Taylor, S. Cousins, *Curr. Eye Res.* **11**, 41 (1992); M. Muhaya *et al.*, *Clin. Exp. Immunol.* **111**, 123 (1998).
14. J. J. Chen, Y. Sun, G. J. Nabel, unpublished observations.
15. J. Raingeaud *et al.*, *J. Biol. Chem.* **270**, 7420 (1995).
16. P. M. Stuart *et al.*, *J. Clin. Invest.* **99**, 396 (1997).
17. N. Nanas, T. F. Molski, C. A. Fernandez, R. I. Sha'afi, *Biochem. J.* **318**, 247 (1996); Y. L. Zu *et al.*, *J. Immunol.* **160**, 7982 (1998); J. A. Nick *et al.*, *J. Clin. Invest.* **99**, 975 (1997).
18. E. Borsou *et al.*, *Biochem. J.* **316**, 167 (1996); D. M. Rose, B. W. Winston, E. D. Chan, L. W. Riches, P. M. Henson, *Biochem. Biophys. Res. Commun.* **238**, 256 (1997); M. Lee, C. Hwang, J. Lee, H. Slavkin, D. Warburton, *Am. J. Physiol.* **273**, L374 (1998); K. Pazdrak, L. Justement, R. Alam, *J. Immunol.* **155**, 4454 (1998).
19. W. H. Daughaday and T. F. Deuel, *Endocrinol. Metab. Clin. North Am.* **20**, 539 (1991).
20. W. C. Liles, J. A. Ledbetter, A. W. Waltersdorph, S. J. Klebanoff, *J. Immunol.* **155**, 2175 (1995).
21. J. Varani *et al.*, *Am. J. Pathol.* **135**, 435 (1989); D. C. Okrent, A. K. Lichtenstein, T. Ganz, *Am. Rev. Respir. Dis.* **141**, 179 (1990).
22. K. Horgan and S. Shaw, *Curr. Protoc. Immunol.* **7**, 4 (1998).
23. Y. Luo and M. E. Dorf, *ibid.* **3**, 20 (1998).
24. Z. Yang *et al.*, *Science* **279**, 1034 (1998).
25. B. A. Arrick *et al.*, *J. Cell Biol.* **118**, 715 (1992).
26. T. M. Kinsella and G. P. Nolan, *Hum. Gene Ther.* **7**, 1405 (1996).

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Identification of Two Distinct Mechanisms of Phagocytosis Controlled by Different Rho GTPases

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The complement and immunoglobulin receptors are the major phagocytic receptors involved during infection. However, only immunoglobulin-dependent uptake results in a respiratory burst and an inflammatory response in macrophages. Rho guanosine triphosphatases (molecular switches that control the organization of the actin cytoskeleton) were found to be essential for both types of phagocytosis. Two distinct mechanisms of phagocytosis were identified: Type I, used by the immunoglobulin receptor, is mediated by Cdc42 and Rac, and type II, used by the complement receptor, is mediated by Rho. These results suggest a molecular basis for the different biological consequences that are associated with phagocytosis.

Phagocytosis is the process by which cells recognize and engulf large particles (>0.5 μm) and is important to host defense mechanisms as well as to tissue repair and morphogenetic remodeling. Two of the best characterized phagocytic receptors in macrophages, the complement receptor 3 (CR3) and Fc gamma receptors (FcγRs), are involved in the uptake of opsonized microorganisms during infection. CR3 binds C3bi on complement-opsonized targets, whereas FcγRs bind to immunoglobulin G (IgG)–coated targets. Phagocytosis by both types of receptors is driven by the reorganization of filamentous actin (F-actin), but the mechanisms of uptake appear to be different (*1, 2*). First, FcγR-mediated uptake is accompanied by pseudopod extension and membrane ruffling, whereas complement-opsonized targets sink into the cell, producing little protrusive activity (*3*). Second, FcγR ligation is accompanied by the activation of the respiratory burst (to produce reactive oxygen species) and by the production of arachidonic acid metabolites and cytokines, such as tumor necrosis factor–α. C3bi-dependent uptake occurs in the absence of any of these proinflammatory signals (*4–6*).

The Rho family of small guanosine triphosphatases (GTPases) is involved in the reorganization of filamentous actin structures in response to extracellular stimuli (*7*). Rho induces the assembly of contractile actomyosin filaments, whereas Rac and Cdc42 control actin polymerization into lamellipodial and filopodial membrane protrusions, respectively (*8, 9*). In addition, these GTPases can affect gene transcription [through the activation of nuclear factor kappa B, through the c-Jun NH_2-terminal kinase (JNK), and through the p38 mitogen-activated protein kinase (MAPK)], and Rac regulates the reduced form of nicotinamide adenine dinucleotide phosphate (NADPH) oxidase enzyme complex that is responsible for the respiratory burst (*10, 11*). We have, therefore, analyzed the relative roles of Rho, Rac, and Cdc42 in FcγR- and CR3-mediated phagocytosis.

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ARTICLES

Fas ligand expression in islets of Langerhans does not confer immune privilege and instead targets them for rapid destruction

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Fas ligand is believed to mediate immune privilege in a variety of tissues, including the eye, testis, and a subset of tumors. We tested whether expression of Fas ligand on pancreatic islets either following adenoviral or germline gene transfer could confer immune privilege after transplantation. Islets were infected with an adenoviral vector containing the murine Fas ligand cDNA (AdFasL), and were transplanted into allogenic diabetic hosts. Paradoxically, AdFasL-infected islets underwent accelerated neutrophilic rejection. The rejection was T cell and B cell independent and required Fas protein expression by host cells, but not on islets. Similarly, transgenic mice expressing Fas ligand in pancreatic β cells developed massive neutrophilic infiltrates and diabetes at a young age. Thus, Fas ligand expression on pancreatic islets results in neutrophilic infiltration and islet destruction. These results have important implications for the development of Fas ligand-based immunotherapies.

Fas ligand (FasL, CD95L) is a type II transmembrane protein of the tumor necrosis factor family that induces cells to send an apoptotic signal to cells expressing Fas (CD95, APO-1)[1,2]. FasL is expressed on a limited number of cell types, including activated T cells, Sertoli cells in the testis, and epithelial cells in the anterior chamber of the eye[3,4]. Fas is a type I transmembrane protein in the tumor necrosis factor receptor/nerve growth factor receptor family[1,2] and is expressed on a variety of cell types, including hepatocytes, activated B and T cells, and neutrophils[5]. Loss of function mutations in the Fas/FasL system result in lymphoproliferation and autoimmunity in both humans[6,7] and mice[8], demonstrating a critical role of this system in T- and B-cell regulation.

The immune privilege of tissues such as the testis[9] and the anterior chamber of the eye[4], has been attributed to local expression of Fas ligand, which presumably acts by inducing apoptosis of invading, Fas-bearing activated T cells. In addition, FasL expression in a subset of tumors may contribute to evasion of immune surveillance[10,11]. A provocative series of reports has suggested that FasL can prevent allograft rejection. For example, the acceptance of transplanted Sertoli cells across MHC barriers in a mouse transplantation model was attributed to FasL expression on those cells[4]. Recently, Lau and co-workers reported that syngeneic myoblasts engineered to express FasL could delay rejection of pancreatic islet allografts when cotransplanted under the kidney capsule[12].

Although the cotransplantation of islets and FasL-expressing myoblasts under the kidney capsule might appear to be an attractive method for improving clinical islet transplantation, islet transplantation under the kidney capsule is not effective in large animals[13,14]. Rather, islet engraftment is best achieved in larger species by infusion into the portal vein with subsequent dispersion throughout the hepatic vasculature[15,16]. After dispersion in the hepatic vasculature, however, myoblast protection of islet allografts may be compromised by a failure of islet cells and myoblasts to colocalize. To circumvent this potential problem, we investigated whether direct FasL expression on transplanted islets could prevent allograft rejection.

We expressed FasL on islets by two distinct methods. First, we transduced murine islets *in vitro* with a replication-defective adenoviral vector expressing a murine FasL cDNA and implanted these islets below the kidney capsule of allogenic mice. As an alternative means of generating FasL-bearing islet cells, we generated transgenic mice that express FasL specifically on islet β cells. Here we report that FasL expression on islets does not confer immune privilege, but instead targets islets for rapid neutrophilic destruction.

Efficient adenoviral transduction of FasL on pancreatic islets

Recombinant adenoviral vectors effectively mediate gene transfer in intact pancreatic islets[17,18]. To assess the efficacy and tissue distribution of adenoviral gene transfer in our experimental system, iso-

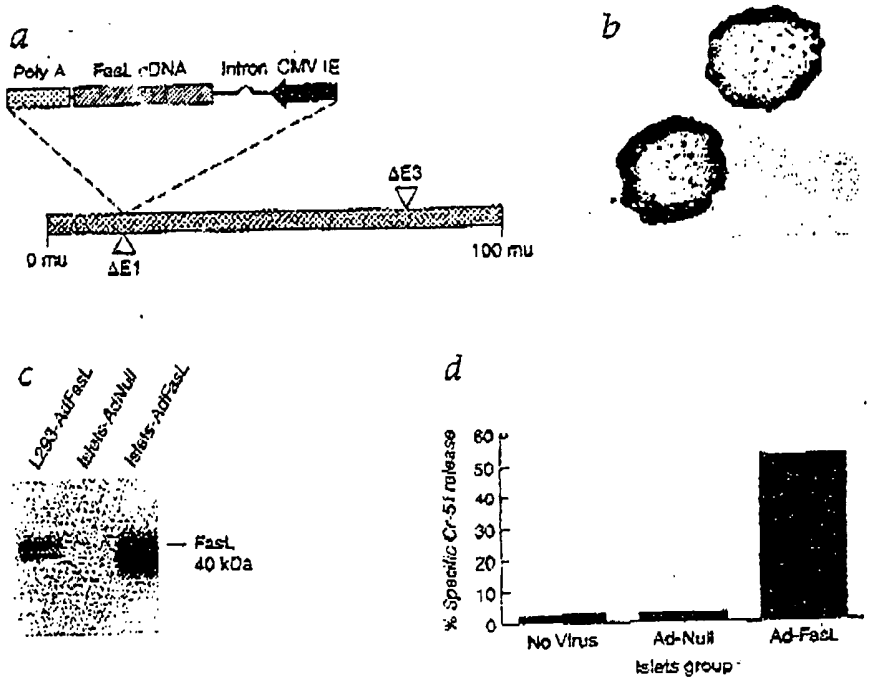


Fig. 1 Expression of functional Fas ligand (FasL) on explanted islets. *a*, The AdFasL vector carries a murine FasL cDNA under the control of the human cytomegalovirus intermediate early promoter (CMV IE). The polyadenylation signal [poly(A)+] is taken from simian virus 40. The vector has deletions in both the E1 and E3 regions of the adenovirus genome. *b*, Histologic sections of X-gal-stained islets 24 h after infection with an adenoviral vector expressing a nuclear targeted β-galactosidase. *c*, Western blot analysis of FasL expression. Immunoreactive FasL is detected in lysates from both 293 cells and islets transduced with AdFasL but not in islets infected with AdNull. *d*, Killing of Fas-bearing targets by AdFasL-transduced cells. ^{51}Cr-labeled, W4 cells (expressing Fas) were mixed with mock-infected islets (no virus), or islets infected with AdNull or AdFasL.

lated mouse islets were transduced with a vector encoding a nuclear targeted β-galactosidase reporter gene (AdRSVnLacZ), stained with X-gal, and embedded in plastic. Examination of histologic sections of the transduced islets revealed β-gal expression solely in the outermost one or two layers of islet cells (Fig. 1*b*), suggesting that the vector does not completely penetrate intact islets. To direct FasL expression on the islets, we generated a replication-defective adenoviral vector containing the murine FasL cDNA (ref. 17) under the control of the cytomegalovirus immediate-early promoter (AdFasL, Fig. 1*a*). The AdFasL vector directed expression of FasL in islets after infection *in vitro*, as assessed by western blot analysis (Fig. 1*c*). Transduction with AdFasL had minimal toxicity to islets for at least 7 days after gene delivery, as judged by light microscopy and by vital staining of cultured islets with dithizone[18]. AdFasL-infected islets specifically killed Fas-expressing W4 lymphoma cells (Fig. 1*d*)[17].

FasL expression accelerates rejection of islet grafts

We assessed whether FasL expression could confer immune privilege and thereby prolong survival of islet allografts. Islets from C3H mice (H-2^k) were transduced with AdFasL and, after a 24-hour incubation *in vitro*, transplanted under the kidney capsule of streptozocin-induced diabetic B6 mice (H-2^b). As controls, islets from C3H mice transduced with an adenoviral vector lacking the FasL expression cassette (AdNull) were transplanted into B6 mice. AdNull-infected islets were rejected in a mean of 9 days (Fig. 2*a*), which is similar to the rejection time for noninfected islets (data not shown). Strikingly, however, FasL-transduced islets underwent accelerated rejection (mean 4 days, $P < 0.001$). This accelerated rejection was not site-specific, as islets expressing FasL also underwent accelerated rejection when infused into the portal vein (data not shown). To investigate the possibility that the accelerated rejection was strain-specific (that is, C3H to

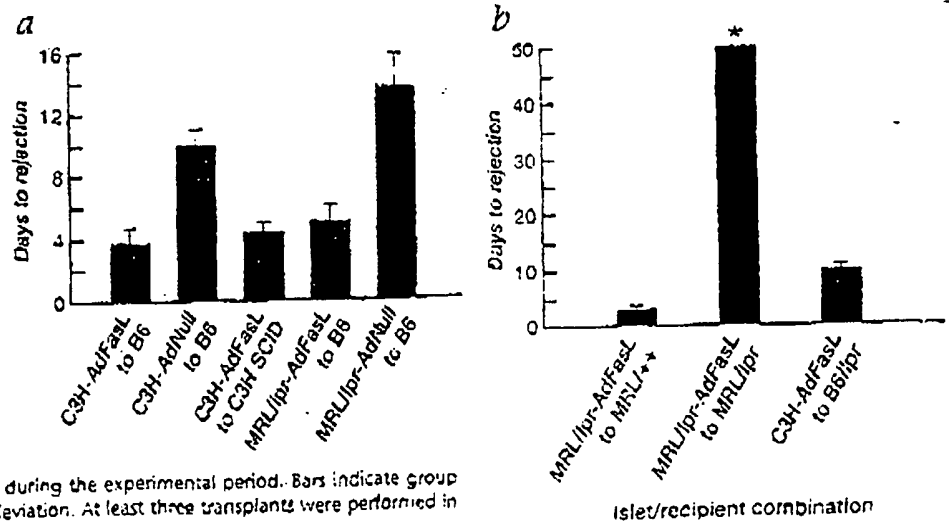


Fig. 2 *a*, Effect of islet FasL expression on graft survival. Experimental groups are listed using the mouse strain used as islet donor, followed by the adenoviral vector used to transduce islets, followed by the recipient strain. All grafts expressing FasL underwent accelerated rejection. *b*, Accelerated islet rejection requires host Fas expression. *No animals in the MRL/lpr-AdFasL to MRL/lpr group underwent graft rejection during the experimental period. Bars indicate group means; error bars indicate one standard deviation. At least three transplants were performed in each group.

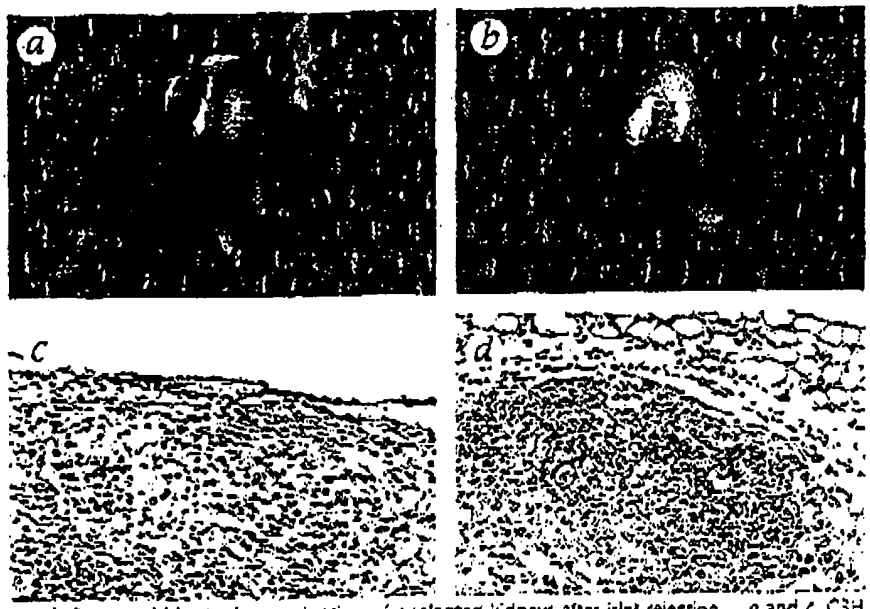


Fig. 3 Gross and histologic examination of explanted kidneys after islet rejection. *a* and *c*, C3H islets infected with AdNull and transplanted into B6 mice, examined 9 days after islet implantation. *b* and *d*, C3H islets infected with AdFasL and transplanted into B6, examined 4 days after islet implantation. Sections in *c* and *d* were stained with esterase; neutrophils stain red.

B6), we tested other combinations of donor and recipient mice. In all combinations tested (including BALB/c (H-2^d) to B6, DBA/2 (H-2^q) to B6, and BALB/c to DBA/2) rejection of FasL-expressing islets was accelerated.

FasL-mediated rejection is T- and B-cell independent

Islet allografts and xenografts survive indefinitely in severe combined immunodeficient (SCID) mice[17], which are deficient in lymphocytes of both the T and B lineages. To assess the role of T and B cells in the accelerated rejection of AdFasL-transduced islets, C3H islets infected with AdFasL were transplanted into diabetic C3H-SCID mice. The islets were rejected in a mean of 4 days, equivalent to the rejection time in fully immunocompetent B6 hosts (Fig. 2*a*).

Islet Fas expression is not required

Normal islets do not express Fas (ref. 20, 21). However, dispersed β cells can be induced to express Fas *in vitro* by interleukin-1β (IL-1β)[18]. In addition, Fas upregulation on β cells *in vivo* has been recently demonstrated in the spontaneous diabetic NOD model[19]. Therefore, the FasL-expressing islet grafts might have failed owing to self-destruction of β cells, mediated via upregulated Fas expression in islet cells after transplantation. To address this possibility, islets from MRL/*lpr* mice (H-2^k), which have a disruption in the *fas* gene[20,21], were transduced with AdFasL and transplanted into diabetic B6 hosts. These islets were rejected as rapidly as AdFasL-infected C3H islets (Fig. 2*a*). Thus, the destruction of FasL-expressing islets is not caused by Fas-mediated apoptosis of β cells.

Host Fas expression is required for accelerated rejection

To investigate whether accelerated rejection of FasL-expressing islets was dependent on Fas expression on host cells, we transplanted FasL-expressing islets into Fas-deficient lpr hosts. MRL/lpr islets infected with AdFasL were accepted indefinitely in MRL/lpr hosts (Fig. 2*b*). In contrast, MRL/lpr islets infected with AdFasL were rejected rapidly in congenic MRL/$^{++}$ hosts, which carry a wild-type *fas* gene (mean 3 days, Fig. 2*b*). Thus, rapid destruction of congenic islets occurs by a Fas-dependent pathway. C3H islets infected with AdFasL were rejected in a mean of 10 days when transplanted into B6/lpr hosts, similar to the rejection time of C3H islets infected with AdNull transplanted into B6 hosts (mean 9 days, Fig. 2, *a* and *b*). Thus, accelerated rejection of both allogenic and syngenic islet grafts expressing FasL is dependent on Fas expression on host cells.

FasL expression induces a rapid neutrophilic infiltrate

On gross examination, rejecting islets of AdNull-islet/Fas$^+$ host and AdFasL-islet/Fas$^-$ host combinations appeared mildly hemorrhagic and inflamed, which is typical of islet allograft rejection (Fig. 3*a*). In contrast, rejecting islets in the AdFasL-islet/Fas$^+$ host combinations had a white, raised appearance, resembling abscess formation (Fig. 3*b*). As expected, histologic examination of explanted kidneys undergoing normal islet allograft rejection showed a lymphocytic infiltrate, with few cells staining positive for granulocyte-specific esterase[22] (Fig. 3*c*). Histologic examination of kidneys explanted from Fas$^-$ hosts after accelerated rejection of AdFasL-infected islets revealed a dense neutrophilic infiltrate. Esterase staining confirmed a neutrophil-predominant infiltrate (Fig. 3*d*). Neutrophilic infiltration was not a late result

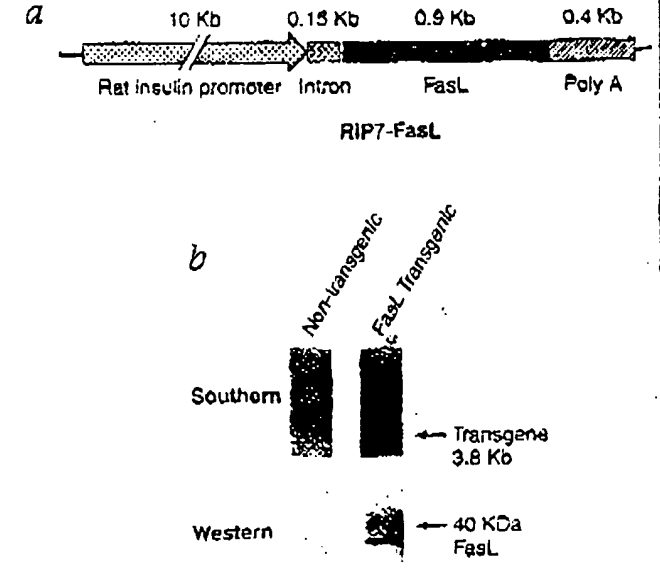
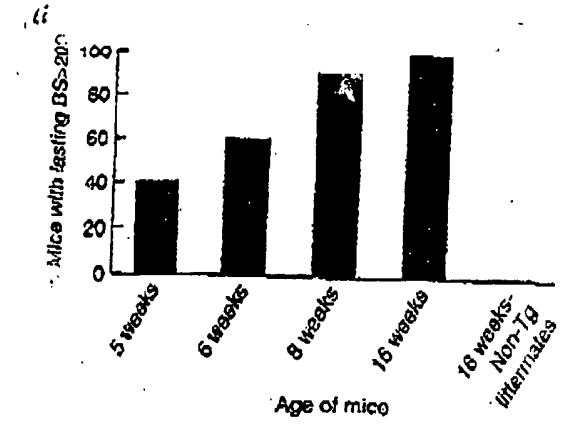


Fig. 4 RIP7-FasL transgenic mice. a, Construct used to generate FasL-transgenic mice. A murine FasL cDNA was spliced to a 10-kb fragment of the rat insulin promoter/enhancer, including the first insulin intron. The polyadenylation signal, poly (A)$^+$, was from the MHC class II E-α gene. b, Southern and western blot analysis of transgenic mice and nontransgenic littermates. Genomic DNA was digested with *Eco*RI and probed with the FasL cDNA. Islets were isolated as described in the Methods section before western blotting.



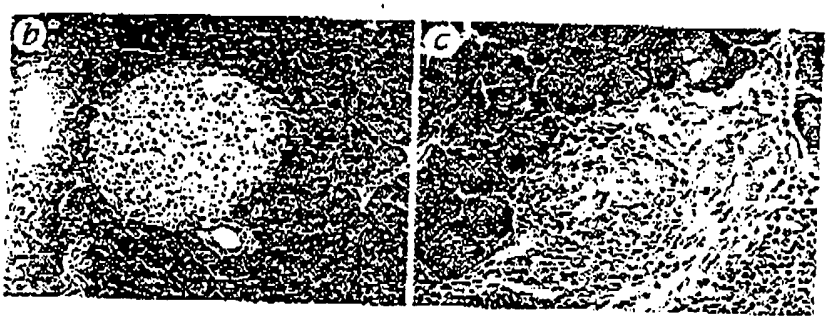


Fig. 5 Diabetes and neutrophilic infiltration of pancreatic islets in transgenic mice with islet cell-specific expression of FasL. *a*, The percentage of transgenic mice with fasting blood sugars (BS) above 200 mg/dl increased steadily over time. Nontransgenic littermates did not develop diabetes. *b*, Esterase stain of pancreas of a nontransgenic mouse at 16 weeks. *c*, Esterase stain of pancreas from a transgenic mouse at 6 weeks.

islet necrosis because neutrophilic infiltrates were seen as ea ly as 24 h after transplantation in the FasL-expressing grafts, when the graft was still functioning and the islet cells appeared viable (data not shown).

Transgenic mice expressing FasL on pancreatic β cells

To study the effect of FasL expression on islets in the absence of possible artifacts resulting from islet isolation, adenovirus-mediated gene transfer, or surgical trauma during transplantation, we generated transgenic mice expressing FasL specifically on pancreatic β cells. The transgenic construct expresses a murine FasL cDNA under the control of a rat insulin promoter/enhancer[25] (RIP7-FasL; Fig 4*a*). The RIP7 vector was chosen because it directs high levels of expressi n in β cells, with excellent fidelity[25]. A transgenic mouse line was generated (RIP7-FasL) containing eight copies of the RIP7-FasL construct. FasL protein expression was demonstrated by western blot analysis of isolated islets (Fig. 4*b*). FasL expression in the RIP7-FasL islets was one-tenth the level expressed on AdFasL-transduced islets, as determined by densitometry.

RIP7-FasL mice develop diabetes and neutrophilic infiltrates in islets

The RIP7-FasL transgenic mice developed diabetes at a young age; 0% of mice were diabetic by 5–6 weeks of age, and 100% of mice were diabetic by 16 weeks (Fig. 5*a*). Histologic examination of pancreata at 5 weeks of age revealed massive infiltration of neutrophils into islets (Fig. 5, *b* and *c*), and older transgenic mice showed progressive scarring and atrophy of islets (data not shown). Thus, germline expression of FasL in pancreatic β cells results in massive neutrophil infiltration, islet destruction, and diabetes.

Discussion

The major findings of this study are as follows: (1) FasL expression on islets of Langerhans results in neutrophilic infiltration and destruction of islets. (2) The mechanism of this accelerated rejection differs from that of alloimmune rejection in that it was T- and B-cell independent. (3) Accelerated rejection of FasL-expressing islets cannot be attributed to Fas-mediated apoptosis of islet cells, because Fas-deficient lpr islets (expressing FasL) underwent a similarly accelerated rejection. (4) Accelerated rejection of FasL-expressing islets was dependent on Fas expression in host tissues.

One possible explanation for our results is that high levels of FasL expression in islet cells results in nonspecific cell dysfunction and death, as reported in association with overexpression of other transgenes in pancreatic β cells, including H-ras and MHC class II antigens[26–28]. If islet cell death resulted merely from overexpression of FasL, the observed neutrophilic infiltration could be a secondary phenomenon. This explanation is, however, inconsistent with both past and present experimental data. Neutrophilic infiltration of islets was not associated with islet dysfunction and destruction in mice overexpressing MHC class II antigen and H-ras (ref. 26–28). Moreover, if islet cell death was a direct result of FasL overexpression, it is unclear why (in the present study) islet rejection occurred only in the presence of Fas expression by host cells.

Recently, Chervonsky *et al.*, showed that Fas expression on β cells can be induced *in vivo* in response to an adoptive transfer of diabetogenic T lymphocytes into 12-week-old NOD mice[21], raising the possibility that FasL expression on islet cells can result in self-induced apoptosis, and perhaps a subsequent neutrophilic infiltration as observed in our experimental system. However, Fas expression in the islet β cells does not appear to be involved in the islet destruction we observed. First, in the C3H/B6 background in which the transgenic mice were generated, a spontaneous infiltration of T cells into the islets of Langerhans is not observed. Thus it is unlikely that Fas expression is induced by T cells as an initiating event in the neutrophilic β-cell destruction found in the FasL transgenic mice. Furthermore, accelerated destruction of islet grafts expressing FasL occurred in SCID mice, which lack T lymphocytes, demonstrating that signals from T lymphocytes are not required for the granulocyte infiltration and islet cell destruction. Second, both lpr islets and wild-type islets (without the *lpr* mutation) were destroyed at similar rates, demonstrating that impairment of Fas expression in islets did not protect them from destruction. Since the *lpr* mutation, caused by the insertion of a transposable element in the *fas* gene, has been demonstrated to permit a low level of Fas expression[29–31], it could be argued that a low level of Fas expression on the lpr islets may lead to self-killing of FasL transduced islets. However, transplantation of FasL-expressing islets containing the wild-type *fas* gene into Fas-deficient lpr hosts completely eliminated the neutrophilic destruction, demonstrating a requirement for Fas expression on host cells rather than on islet cells. We there-

fore conclude that the destruction of pancreatic islet cells observed in our study is not due to an upregulation of Fas on pancreatic β cells and an autocrine Fas–FasL-mediated suicide.

Our data are most consistent with the hypothesis that FasL expression results in the recruitment of neutrophils into islet tissue, leading to islet destruction. This hypothesis is supported by the data of Seino *et. al.*, which showed that tumor lines stably transfected with FasL undergo neutrophil-dependent destruction after subcutaneous implantation[29]. We have similarly found rapid neutrophilic infiltration of several FasL-expressing cell lines implanted under the kidney capsule (data not shown). Lau and co-workers[13] did not observe a neutrophilic response to FasL expressing myoblasts, which in their study prolonged islet allograft survival. In contrast, we have found that syngeneic myoblasts expressing FasL undergo rapid neutrophilic destruction when implanted under the kidney capsule (S.-M.Kang, A. Hofmann, D. Lee, P.G. Stock, H. Blau, manuscript in preparation). Moreover, coimplantation of FasL-expressing myoblasts with unmanipulated allogenic islets accelerated rejection. Further investigations will be required to resolve these divergent findings.

Our data also indicate that neutrophil recruitment is dependent on islet expression of FasL and host expression of Fas. However, the precise molecular mechanism of neutrophil recruitment in this setting remains to be elucidated, in particular, the identification of the cell type in which Fas expression is critical for the phenotype. This mechanism is of substantial interest because abrogation of neutrophil recruitment might unmask the ability of FasL to protect allografts from T cell-mediated rejection. In this regard, FasL mediates upregulation of IL-8 expression in a colon carcinoma cell line, HT-29 (ref. 30), and in synoviocytes[31]. IL-8 is critical for neutrophil chemotaxis and activation[32]. It is therefore possible that expression of FasL results in upregulation of IL-8 expression in surrounding tissues, leading to recruitment of neutrophils[33,34]. However, it is also possible that soluble FasL, which is released from the cell surface by matrix metalloprotease cleavage of membrane-bound FasL (ref. 34–36), can act directly as a neutrophil chemotactic factor. Consistent with this hypothesis, Fas is highly expressed on neutrophils[37–39].

If FasL expression results in neutrophil chemotaxis, by what mechanism do FasL-expressing tissues such as the eye[9] and the testis[8] escape neutrophil-mediated destruction? One possibility is that these sites are intrinsically less susceptible to neutrophil chemotaxis, perhaps because of low or absent IL-8 production in response to FasL. A second possibility is that the level of FasL expression at these sites is below a threshold at which stimuli for neutrophil infiltration are generated. Relevant to this possibility, we have recently generated a second transgenic mouse line that expresses approximately one-fourth the levels of FasL on β cells as compared with the RIP7-FasL line described above. This second line of mice expresses functional FasL on β cells, as judged by islet cytotoxicity to W4 cells; however, the mice do not develop neutrophilic islet infiltrates or diabetes. In preliminary transplantation experiments, the survival of islets grafted from this line of transgenic mice was similar to survival of islet grafts from nontransgenic littermates (data not shown). Thus, the level of FasL expression on cells of these mice appears insufficient to cause neutrophil accumulation, but also does not confer immune privilege. Independently, Allison *et al.* have generated a FasL transgenic mouse line using a shorter version of the rat insulin promoter[40]. These mice also develop neutrophilic infiltrates in the islets, but do not develop diabetes. Survival of islet transplants from these mice are neither shortened nor prolonged compared with islet transplants from nontransgenic littermates. Thus, they appear to have a phenotype intermediate between those seen in our two lines, which may suggest a dose–response relationship between FasL expression levels and the intensity of neutrophilic infiltration. However, FasL-mediated protection was not seen in any of the mouse lines. A third potential explanation for the lack of neutrophilic infiltrates in the eye and testis is low or absent expression of the matrix metalloprotease(s) that cleave FasL to soluble FasL (ref. 34–36). In the absence of cleavage and release of soluble FasL, the action of FasL would be limited to cells in direct contact with FasL-expressing cells.

Our findings are relevant to the development of FasL-based strategies to modify immune responses. Although other studies suggest a role of FasL in preventing allograft rejection[4,13], we have found in a clinically relevant transplant model that FasL acts as a proinflammatory factor that accelerates, rather than prevents, graft loss. This proinflammatory function of FasL will require further characterization and modulation before FasL-based immunotherapies can be applied.

Methods

Adenoviral vectors. AdFasL: The 940-bp coding region of the murine FasL cDNA (ref. 17; kindly provided by S. Nagata) was excised from pBL-MFLW4 by *Xba*I digestion and was ligated into the *Xba*I site of pCI (Promega, Madison, WI), which contains the CMV immediate-early enhancer/promoter, a chimeric intron, and the SV-40 polyadenylation signal (Fig. 1*a*). A *Fsp*I/*Bam*HI fragment containing the pCI-FasL expression cassette was then cloned into *Bam*HI/*Eco*RV-restricted pΔE1sp1A (Microbix Biosystems, Toronto, Ontario; a left-end adenovirus shuttle plasmid containing Ad5 sequences from map units 0 to 15.8 and a 3.2 kb E1 deletion) to generate pΔE1sp1A-FasL. AdFasL was generated by homologous recombination in 293 cells following cotransfection of linearized pΔE1sp1A-FasL and the 33 kb *Cla*I fragment of Ad-dl327 (an E3-deleted Ad)[41]. AdRSVnLacZ: The construction of AdRSVnLacZ, an E1- and E3-deleted recombinant adenovirus vector containing a nuclear targeted *Escherichia coli* β-galactosidase gene under the control of the RSV long terminal repeat promoter, has been described in detail previously[42]. AdNull: This virus contains the E1 and E3 deletions identical to those in AdFasL, but it does not contain a transgene. AdNull was generated by homologous recombination between the 33-kb *Cla*I fragment of Ad-dl327 and linearized pΔE1sp1A. All vectors were amplified in 293 cells, purified, and titered as described[43]. Viral stocks were free of replication-competent virus as determined by assays capable of detecting replication-competent virions at a frequency of 1 in 10^7.

Cytotoxicity assays. Fifty islets were mixed with 1×10^4 Fas-expressing ^{51}Cr-labeled W4 cells[17] (kindly provided by S. Nagata) in a volume of 200 µl in wells of a 96-well V-bottom plate. After overnight incubation, radioactivity in the supernatant was measured by scintillation counting (Wallac, Gaithersburg, Maryland). Specific killing was calculated as [(experimental release – spontaneous release)/(maximal release – spontaneous release)] × 100. All groups were run in triplicate.

Transplants. Murine islets were isolated by collagenase digestion and purification on dextran gradients, followed by hand-picking, as described[44]. Purified islets were cultured for 2 h in islet medium [10% FCS in RPMI (Life Technologies, Grand Island, NY)] before infection. Islets were then transferred to reduced serum medium (2% FCS in RPMI). Adenovirus (AdNull or AdFasL) was added at a multiplicity of infection of 2.25, with the assumption that each islet contains approximately 1×10^3 cells. After a 2-h incubation, the islets were transferred into islet medium and cultured for an additional 20–24 h before transplantation. Diabetes (defined as at least two consecutive days with blood glucose >350 mg/dl) was induced in recipient mice by intraperitoneal injection of 300 mg/kg of streptozocin. Each recipient received 800–1000 islets under the left renal capsule via a 27 gauge needle. Rejection was defined as blood sugars >250 mg/dl on two consecutive days, following at least one post-transplant glucose <150 mg/dl. A min

imum of three independent transplants was performed for each islet/host combination.

Western blot. Samples were lysed in RIPA buffer (PBS with 1% vol/vol NP-40, 0.5% wt/vol sodium deoxycholate, 0.1% wt/vol SDS), and were resolved on a 12% (wt/vol) SDS polyacrylamide gel. Duplicate gels were run and stained with Coomassie blue to confirm approximately equivalent protein loading between samples. Proteins were transferred to nitrocellulose. After blocking with 20% nonfat dry milk in Tris-buffered saline with 0.1% (vol/vol) Tween 20, FasL protein was detected with a polyclonal rabbit anti-FasL antibody (N-20, Santa Cruz Biotechnology, Santa Cruz, CA), per the manufacturer's instructions. Bound antibody was detected with peroxidase-coupled anti-rabbit IgG and enhanced chemiluminescence (ECL; Amersham, Arlington Heights, IL). A densitometer (Bio-Rad, Hercules, CA), was used to compare signal intensity.

Transgenic mice. The murine FasL cDNA was adapted with *ClaI* ends via polymerase chain reaction (PCR), using the primers dTCGGGATCCATCGATAAGGAAACCCTTTCCTGG and dCCGAATTCATCGATTCCTGGTGCCCATGATAAAG. The PCR product was ligated into the *ClaI* site of the RIP7 vector[25]. After the FasL cDNA was sequenced, the construct was linearized and injected into C3H/B6 F, embryos. Transgenic mice were identified by Southern blot analysis, and copy number was determined by densitometry. Mice were bred into the C3H background for one to two generations before analysis.

Histology. Explanted tissues were fixed in 10% neutral buffered formalin, embedded in paraffin, and processed for hematoxylin and eosin staining or a granulocyte-specific stain that employs naphthol AS-D-chloroacetate as a substrate for esterase[26]. Granulocytes appear red with the esterase stain. Expression of β-galactosidase in intact islets fixed in 2% formaldehyde/0.2% glutaraldehyde was detected with 5-bromo-4-chloro-3-indolyl-D-galactopyranoside (X-gal). X-gal-stained islets were embedded in plastic, cut in 5-μm-thick sections, and counterstained with nuclear fast red.

Statistics. Results are graphed as the mean ± 1 s.d. Differences were assessed using unpaired Student's t-test with the aid of SigmaStat software (Jandel Scientific, San Rafael, CA).

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1. Nagata, S. Fas and Fas ligand: A death factor and its receptor. *Adv. Immunol.* 57, 129–144 (1994).
2. Krammer, P.H. *et al.* The role of APO-1-mediated apoptosis in the immune system. *Immun. Rev.* 142, 175–191 (1994).
3. Nagata, S. & Golstein, P. The Fas death factor. *Science* 267, 1449–1456 (1995).
4. Nagata, S. Fas ligand and immune evasion. *Nature Med.* 2, 1306–1307 (1996).
5. Fisher, G.H. *et al.* Dominant interfering fas gene mutations impair apoptosis in a human autoimmune lymphoproliferative syndrome. *Cell* 81, 935–946 (1995).
6. Rieux-Laucat, F. *et al.* Mutations in Fas associated with human lymphoproliferative syndrome and autoimmunity. *Science* 268, 1347–1349 (1995).
7. Nagata, S. & Suda, T. Fas and Fas ligand: lpr and gld mutations. *Immun. Today* 16, 39–43 (1995).
8. Bellgrau, D. *et al.* A role for CD95 ligand in preventing graft rejection. *Nature* 377, 630–632 (1995).
9. Griffith, T.S., Brunner, T., Fletcher, S.M., Green, D.R. & Ferguson, T.A. Fas ligand-induced apoptosis as a mechanism of immune privilege. *Science* 270, 1189–1192 (1995).
10. Strand, S. *et al.* Lymphocyte apoptosis induced by CD95 (APO-1/Fas) ligand-expressing tumor cells — A mechanism of immune evasion? *Nature Med.* 2, 1361–1366 (1996).
11. Hahne, M. *et al.* Melanoma cell expression of Fas (Apo-1/Cd95) ligand — implications for tumor immune escape. *Science* 274, 1363–1366 (1996).
12. Lau, H.T., Yu, M., Fontana, A. & Stoeckert, C.J., Jr. Prevention of islet allograft rejection with engineered myoblasts expressing FasL in mice. *Science* 273, 109–112 (1996).
13. Hesse, U.J., Sutherland, D.E., Gores, P.F., Sitges-Serra, A. & Najarian, J.S. Comparison of splenic and renal subcapsular islet autografting in dogs. *Transplantation* 41, 271–274 (1986).
14. Sutherland, D.E. Pancreas and islet transplantation. I. Experimental studies. *Diabetologia* 20, 161–185 (1981).
15. Becker, T.C., Beltrandelrio, H., Noel, R.J., Johnson, J.H. & Newgard, C.B. Overexpression of hexokinase I in isolated islets of Langerhans via recombinant adenovirus. *J. Biol. Chem.* 269, 21234–21238 (1994).
16. Csete, M.E. *et al.* Efficient gene transfer to pancreatic islets mediated by adenoviral vectors. *Transplantation* 59, 263–268 (1995).
17. Takahashi, T. *et al.* Generalized lymphoproliferative disease in mice, caused by a point mutation in the fas ligand. *Cell* 76, 969–976 (1994).
18. Hansen, W.A. *et al.* Supravital dithizone staining in the isolation of human and rat pancreatic islets. *Diabetes Res.* 10, 53–57 (1989).
19. Shiroki, R. *et al.* Human peripheral blood lymphocyte reconstituted severe combined immunodeficient (hu-PBL-SCID) mice: A model for human islet allograft rejection. *Transplantation* 57, 1553–1562 (1994).
20. Yamada, K. *et al.* Mouse islet cell lysis mediated by interleukin-1-induced Fas. *Diabetologia* 39, 1306–1312 (1996).
21. Chervonsky, A. *et al.* The role of Fas in autoimmune diabetes. *Cell* 89, 17–24 (1997).
22. Adachi, M., Watanabe-Fukunaga, R. & Nagata, S. Aberrant transcription caused by the insertion of an early transposable element in an intron of the Fas antigen gene of lpr mice. *Proc. Natl. Acad. Sci. USA* 90, 1756–1760 (1993).
23. Adachi, M. *et al.* Targeted mutation in the fas gene causes hyperplasia in peripheral lymphoid organs and liver. *Nature Genet.* 11, 294–300 (1995).
24. Moloney, W.C., McPherson, K. & Fliegelman, L. Esterase activity in leukocytes demonstrated by the use of naphthol AS-D chloroacetate substrate. *J. Histochem. Cytochem.* 8, 200–207 (1960).
25. Jolicoeur, C., Hanahan, D. & Smith, K.M. T-cell tolerance toward a transgenic beta-cell antigen and transcription of endogenous pancreatic genes in thymus. *Proc. Natl. Acad. Sci. USA* 91, 6707–6711 (1994).
26. Efrat, S. Sexual dimorphism of pancreatic beta-cell degeneration in transgenic mice expressing an insulin-ras hybrid gene. *Endocrinology* 128, 897–901 (1991).
27. Lo, D. *et al.* Diabetes and tolerance in transgenic mice expressing class II MHC molecules in pancreatic beta cells. *Cell* 53, 159–168 (1988).
28. Sarvetnick, N., Liggitt, D., Pitts, S.L., Hansen, S.E. & Stewart, T.A. Insulin-dependent diabetes mellitus induced in transgenic mice by ectopic expression of class II MHC and interferon-gamma. *Cell* 52, 773–782 (1988).
29. Saino, K.-I., Kayagaki, N., Okumura, K. & Yagita, H. Antitumor effect of locally produced CD95 ligand. *Nature Med.* 3, 165–170 (1997).
30. Abreu-Martin, M.T., Vidrich, A., Lynch, D.H. & Targan, S.R. Divergent induction of apoptosis and IL-8 secretion in HT-29 cells in response to TNF-alpha and ligation of Fas antigen. *J. Immunol.* 155, 4147–4154 (1995).
31. Sakina, C. *et al.* Fas-mediated stimulation induces IL-8 secretion by rheumatoid arthritis synoviocytes independently of Cpp32-mediated apoptosis. *Biochem. Biophys. Res. Commun.* 228, 14–20 (1996).
32. Baggiolini, M. & Clark-Lewis, I. Interleukin-8, a chemotactic and inflammatory cytokine. *FEBS Lett.* 307, 97–101 (1992).
33. Cacalano, G. *et al.* Neutrophil and B cell expansion in mice that lack the murine IL-8 receptor homolog. *Science* 265, 682–684 (1994).
34. Tanaka, M., Suda, T., Takahashi, T. & Nagata, S. Expression of the functional soluble form of human fas ligand in activated lymphocytes. *EMBO J.* 14, 1129–1135 (1995).
35. Mariani, S.M., Matiba, B., Baumler, C. & Krammer, P.H. Regulation of cell surface APO-1/Fas (CD95) ligand expression by metalloproteases. *Eur. J. Immunol.* 25, 2303–2307 (1995).
36. Kayagaki, N. *et al.* Metalloproteinase-mediated release of human Fas ligand. *J. Exp. Med.* 182, 1777–1783 (1995).
37. Iwai, K. *et al.* Differential expression of bcl-2 and susceptibility to anti-Fas-mediated cell death in peripheral blood lymphocytes, monocytes, and neutrophils. *Blood* 84, 1201–1208 (1994).
38. Liles, W.C., Kiener, P.A., Ledbetter, J.A., Aruffo, A. & Klebanoff, S.J. Differential expression of Fas (CD95) and Fas ligand on normal human phagocytes: Implications for the regulation of apoptosis in neutrophils. *J. Exp. Med.* 184, 429–440 (1996).
39. Liles, W.C. & Klebanoff, S.J. Regulation of apoptosis in neutrophils — Fas track to death? *J. Immunol.* 155, 3289–3291 (1995).
40. Allison, J., Georgiou, H.M., Strasser, A., & Vaux, D. Transgenic expression of CD95 ligand on islet beta cells induces a granulocytic infiltration, but does not confer immune privilege upon islet allografts. *Proc. Natl. Acad. Sci. USA* (in the press).
41. Ginsberg, H.S. *et al.* Role of early region 3 (E3) in pathogenesis of adenovirus disease. *Proc. Natl. Acad. Sci. USA* 86, 3823–3827 (1989).
42. Dong, G., Schullick, A.H., DeYoung, M.B. & Dichek, D.A. Identification of a cis-acting sequence in the human plasminogen activator inhibitor type-1 gene that mediates transforming growth factor-beta1 responsiveness in endothelium in vivo. *J. Biol. Chem.* 271, 29969–29977 (1996).
43. Graham, F.L. & Prevec, L. *Methods in Molecular Biology*. 109–128 (Humana Press, Clifton, NJ, 1991).
44. Osoria, R.W., Ascher, N.L., Melzer, J.S. & Stock, P.G. β2-Microglobulin gene disruption prolongs murine islet allograft survival in NOD mice. *Trans. Proc.* 26, 752 (1994).